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HOW DO CHEMOTHERAPEUTIC AGENTS DAMAGE THE OVARY?

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**Submitted for PhD Thesis Examination at the University of
Edinburgh**

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Declaration

I declare that this thesis has been composed by myself and has not been submitted for any previous degree. The work described here is my own and all work by other authors is acknowledged. I also acknowledge any assistance received.

Stephanie Morgan

Abstract

Chemotherapy treatment in premenopausal women has been linked to premature ovarian failure (POF), and hence infertility, through ovarian follicle loss. The exact mechanisms that lie behind this loss are unclear and so the action of two commonly used chemotherapeutic agents were compared here. Cisplatin is a DNA cross-linking agent commonly used in the treatment of ovarian, lung and bladder cancers, while the anthracycline doxorubicin is commonly used to treat leukaemia and breast cancer. Neonatal mouse ovaries were cultured *in vitro* and exposed to cisplatin or doxorubicin in order to determine their effects on primordial and early growing follicles. Both drugs caused a dose dependant follicle loss but targeted different cell types. Cisplatin caused a significant increase in follicles with unhealthy oocytes; furthermore primary stage follicles were the follicle class most affected (up to 98% classified as unhealthy compared with 13% in control, $p < 0.001$). In contrast, doxorubicin caused a significant increase in follicles with unhealthy granulosa cells and affected all follicle stages present. When the mechanism of cell death was further investigated, apoptosis was the main pathway through which these drugs cause ovarian cell death. Doxorubicin in particular caused a significant increase in apoptosis of ovarian somatic cells including the granulosa cells and stroma. Imatinib mesylate, a tyrosine kinase inhibitor which is also used as a chemotherapeutic agent, has been implicated as a potential therapy to block the ovotoxic effects of cisplatin. Results here confirm this finding (29% of follicles classified as unhealthy in the cisplatin only group compared to 8% in the cisplatin and imatinib co-treatment group, $p < 0.001$) and found further, that imatinib was unable to protect against doxorubicin-induced damage (28% of follicles classified as unhealthy in the

doxorubicin treated group compared to 19% in the doxorubicin and imatinib co-treatment group). Imatinib treatment alone in newborn ovaries caused a significant increase in the number of follicles present at the end of culture compared to control (402 ± 43 in the imatinib group compared to 188 ± 34 in control, $p < 0.001$), which is likely due to an effect on follicle formation. In conclusion, the work presented in this thesis demonstrates drug specific actions of cisplatin and doxorubicin on the mouse ovary. This suggests that any therapy designed to confer ovarian protection in the future may have to be tailored to be drug specific.

Publications arising from this work

Morgan, S., Anderson, R. A., Gourley, C., Wallace, W. H. B., and Spears, N. (2012).
How do chemotherapeutic agents damage the ovary? *Human Reproduction Update*
18, 525-35.

Morgan, S., Lopes, F., Gourley, C., Anderson, R. A., and Spears, N. (2013).
Cisplatin and Doxorubicin Induce Distinct Mechanisms of Ovarian Follicle Loss;
Imatinib Provides Selective Protection Only against Cisplatin. *PloS One* **8**, e70117.

Abstracts for poster presentations

Stephanie Morgan, Richard Anderson and Norah Spears.

The effect of cisplatin on the ovary

Fertility 2010

Stephanie Morgan, Richard Anderson and Norah Spears

The effect of cisplatin and doxorubicin on the ovary.

Reproductive Function and Dysfunction, 2011.

Stephanie Morgan, Richard Anderson and Norah Spears.

Cisplatin and doxorubicin cause ovarian cell death through apoptosis not autophagy.

Society for the Study of Reproduction, 2013.

Assistance given throughout this investigation

All work detailed throughout this thesis was conducted by myself with the following exceptions:

Chapter 3: Assessment of follicle distribution over the 6 day newborn mouse ovary culture was done by Federica Lopes. I was helped in the setting up of the bovine cultures by Marie McLaughlin.

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Abbreviations

5FU. 5-flourouracil

6MP. 6-mercaptopurine

6TG. 6-thioguanine

ABL. Abelson murine leukemia viral oncogene homolog

ABVD. Adriamycin, bleomycin, vinblastin, dacarbazine

ABVE. Adriamycin, bleomycin, vinblastine, etoposide

AC. Adriamycin, cyclophosphamide

ACTD. Adriamycin, cyclophosphamide, actinomycin D

ADE-GMTZ. Arabinoside-C, Daunorubicin, Etoposide, Gemtuzumab
ozogamicin

ADP. Adenosine Diphosphate

AI. Arabinoside cytoside, idarubicin

AIE. Arabinoside cytosine, idarubucin, etoposide

AMH. Anti Müllerian Hormone

AMP. Adenosine Monophosphate

APAF-1. Apoptotic protease activating factor 1

AS101. Ammonium tri-chloro(dioxoethylene-O,O'-)tellurate

ATG7. Autophagy-related protein 7

ATG13. Autophagy-related protein 13

ATP. Adenosine Triphosphate

BCL2. B-cell lymphoma 2

BEA. Bleomycin, etoposide, adriamycin

BEACOPP. Bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, prednisone.

BMP-15. Bone Morphogenetic Protein-15

BSA. Bovine Serum Albumin

CAF. Cyclophosphamide, adriamycin, 5-fluorouracil

CC3. Cleaved Caspase 3

cDNA. Complementary Deoxyribonucleic Acid

CHOP. Cyclophosphamide, doxorubicin, vincristine, prednisone

CMF. Cyclophosphamide, Methotrexate and 5-fluorouracil

COAP. Cyclophosphamide, vincristine, cytosine arabinoside, prednisone

COPP. Cyclophosphamide, oncovin, procarbazine, prednisone

CVPP. cyclophosphamide, vincristine, procarbazine, prednisone; DNR:

Daunorubicin

DAB. 3,3'-Diaminobenzidine

DNA. Deoxyribonucleic Acid

DNR. Daunorubicin

E. Embryonic Day

E-PIE. Etoposide, cisplatin, I-fosphamide

FBS. Foetal Bovine Serum

FOXO3a. Forkhead box O3

FSH. Follicle Stimulating Hormone

GAPDH. Glyceraldehyde 3-phosphate dehydrogenase

GDF-9. Growth Differentiation Factor-9

GMALL. Methotrexate, cyclophosphamide, vincristine, daunorubicin,
asparaginase, cytarabine, 6-mercapto-purine, etoposide

GMP. Guanosine Monophosphate

GnRH- Gonadotrophin Releasing Hormone

GVBD. Germinal Vesicle Breakdown

HAM. High dose arabinoside cytosine, mitoxantrone

hCG. Human Chorionic Gonadotrophin

I-Fos. I-fosfamide

ICM. Inner Cell Mass

IL. Interleukin

IVF. In vitro Fertilisation

IVM. In Vitro Maturation

JET. Carboplatin, etoposide

LH. Luteinizing Hormone

MECC. Melphalan, etoposide, citarabin, carmustine

MII. Meiotic arrest, stage two

MINE EASHAP. Mesna, ifosfamide, mitoxantrone, etoposide, cytosine arabinoside, cis-platinum and steroids

MPF. Maturation Promoting Factor

mRNA. Messenger Ribonucleic Acid

MTX. Methotrexate

MVH. Mammalian Vasa Homolog

NAD. Nicotinamide Adenine Dinucleotide

NER. Nucleotide Excision Repair

P. Postnatal day

P. Procarbazine

PBS. Phosphate Buffered Saline

PCR. Polymerase Chain Reaction

PDGF. Platelet Derived Growth Factor

PMF. Primordial Follicles

PMSG. Pregnant Mare Serum Gonadotrophin

PND. Postnatal Day

POF. Premature Ovarian Failure

Pr. Prednisone

PTEN. Phosphatase and Tensin Homolog

PVD. Prednisone, vincristine, doxorubicin

RNA. Ribonucleic acid

RT. Reverse Transcriptase

S1P. Sphingosine-1-phosphate

SDS. Sodium dodecyl sulphate

SEM. Standard error of the mean

SCF. Stem Cell Factor

STWS. Scotch Tap Water Substitute

TE. Trophectoderm

TEMED. N,N,N',N'-Tetramethylethylenediamine

TNF. Tumour necrosis factor

TSC-2. Tuberous Sclerosis 2

TUNEL. Terminal deoxynucleotidal transferase dUTP nick end labelling

ULK1. Unc-51 like autophagy activating kinase 1

V. Vincristine

VACOP-B. Doxorubicin, cyclophosphamide, vincristine, bleomycin,
etoposide

VC. Vincristine, cyclophosphamide

VCAIE. Vincristine, cyclophosphamide, arabinosidecytosine, idarubicin,
etoposide

VMTX. Vincristine, methotrexate

ZP. Zona Pellucida

CHAPTER 1

GENERAL INTRODUCTION

1.1 General Overview of the Ovary

By the time of birth in humans, the ovary contains all the germ cells required for the reproductive lifespan of the female. These germ cells are maintained within primordial follicles, a functional unit which consists of a germ cell, oocyte, surrounded by a few flattened pregranulosa cells. At birth, there are approximately 1-2 million primordial follicles within the ovary, by the onset of puberty there are 300,000 remaining. These primordial follicles can remain in a dormant state for the full reproductive lifespan before being activated to grow. At any one time, small cohorts of these follicles are recruited out of the resting primordial pool and are activated to grow. This process is continuous until there are no follicles remaining; when fewer than a thousand remain, this is defined as the onset of the menopause (Wallace and Kelsey, 2010). In this thesis, the mouse was used as a model and so is the species that will be referred to throughout.

1.1.1 Follicle Formation

Primordial germ cells (oocyte precursors) arise during embryonic gastrulation and actively migrate through the hindgut epithelium and dorsal mesentery to the site of the genital ridge, arriving there around embryonic day (E) 11.5 (Molyneaux *et al*, 2001). The primordial germ cells are then referred to as oogonia and undergo mitotic divisions, forming germ cell nests (also called cysts) in which the cells are connected through intercellular bridges (Pepling, 2012). These germ cell nests are surrounded by somatic cells which invade the ovary and are generally thought to be derived from the ovarian surface epithelium (Sawyer *et al*, 2002). The oogonia divide mitotically until around E13.5 (in humans this corresponds to around week 13 of gestation), after

which they enter prophase 1 of meiosis, arrest in the diplotene phase and enlarge; thereafter they are referred to as oocytes. The somatic cells surrounding the nests closely associate with the oocytes and differentiate into follicular granulosa cells. Germ cell nest breakdown occurs around the time of birth in mice during which oocytes separate and are surrounded by pregranulosa cells to form primordial follicles. In humans, primordial follicles are first seen between 16 and 21 weeks of gestation (Konishi *et al*, 1986). Only a third of the oocytes which undergo cyst breakdown survive; the rest die through a process of programmed cell death. Once granulosa cells have surrounded the oocyte, the follicle is encompassed by a basement membrane.

Oocyte loss during germ cell nest breakdown is substantial and postulated to be a quality control mechanism whereby the 'best' oocytes are preferentially selected to become primordial follicles (Tingen *et al*, 2009). The mechanism by which so many oocytes are lost is likely to be apoptosis with the bulk of evidence implicating the Bcl2 (B-cell lymphoma 2) family as being key regulators of this process (De Felici *et al*, 1999). However recent evidence has also implicated autophagy as an important cell death mechanism in cyst breakdown (De Felici *et al*, 2008).

Due to the fact that the oocytes have entered meiosis, the population of primordial follicles is fixed by the time of birth and will be continuously depleted over the reproductive lifespan. Although this has been recently challenged with studies suggesting the existence of female germline stem cells (Johnson *et al*, 2004; White *et al*, 2012; Zou *et al*, 2009), the conventional view is that the primordial follicle reserve is finite, meaning that any toxic insult to this pool is likely irrevocable. The existence of these stem cells is still controversial and in my opinion, there is

currently no robust evidence for the existence of germ stem cells in the postnatal ovary which function *in vivo*.

1.1.2 Follicle Activation

The mechanism by which primordial follicles activate, while still not fully understood, has been largely elucidated following *in vitro* work which demonstrated widespread activation of bovine primordial follicles in culture due to the removal of inhibitory signals (Wandji *et al*, 1996) and several rodent gene knockout models. The activation of primordial follicles is orchestrated through a variety of local paracrine factors including several inhibitory signals such as Foxo3a (Forkhead box O3, Castrillon *et al*, 2003), Tsc-2 (Tuberous sclerosis 2, Adhikari *et al*, 2009), p27 (Rajareddy *et al*, 2007) and PTEN (Phosphatase and tensin homolog, Reddy *et al*, 2008). As primordial follicles activate, the flattened pre-granulosa cells that surround the oocyte proliferate and become cuboidal to form a single layer; this is defined as a primary follicle. During this transition, the oocyte increases in size and the zona pellucida forms. The zona pellucida is a matrix composed of four glycoproteins, ZP1, 2, 3, and 4 which envelops the oocyte (Rankin *et al*, 2000), with the granulosa cells maintaining their close association with the oocyte through gap junctions which penetrate the zona (Carabatsos *et al*, 2000). In the mouse only ZP1, 2, and 3 are expressed in the zona pellucida (Bleil and Wassarman, 1980).

The growth of primary follicles is largely gonadotrophin-independent and is likely reliant on a variety of paracrine and autocrine signals. When isolated, primary follicles degenerate rapidly in culture, indicating a reliance on the other cell types in the ovary including stromal cells as well as other follicles (Hornick *et al*, 2013).

Follicles which are growing are often surrounded by other growing follicles, perhaps indicating that they have a stimulatory effect upon each other's growth (Da Silva-Buttkus *et al*, 2009).

1.1.3 Ovarian Reserve

The ovarian reserve describes the population of primordial follicles remaining in the ovary. The conventional view is that this reserve is finite once established, and is depleted over time by both degeneration and the activation of follicles into the growing population. The factors that govern these processes therefore are vital to the maintenance of the primordial follicle reserve and hence a females reproductive lifespan (Kerr *et al* 2013). Major factors which are known to be involved in these processes are listed below (Table 1.1).

Table 1.1. Summary of the major factors involved in the maintenance of the ovarian reserve. This involves factors/pathways which regulate primordial germ cell number, primordial follicle formation and follicle activation.

	Germ cell numbers	Primordial follicle formation	Follicle activation	Reference
FIGa	x	Required for	x	Soyal <i>et al</i> 2000
<i>Dazla</i>	Increases/is necessary for maintenance of	x	x	Ruggiu <i>et al</i> 1997
<i>Grem1</i>	Increases	Accelerates	x	Myers <i>et al</i> 2011
<i>Bax</i>	Reduces	x	x	Greenfeld <i>et al</i> 2007
<i>Bcl2</i>	Increases	x	x	Flaws <i>et al</i> 2001
Activin A	Increased survival	x	x	Martins da Silva <i>et al</i> 2004
Neurotrophins	Reduces	x	x	Spears <i>et al</i> 2003
<i>Pten</i>	x	x	Suppressor	Reddy <i>et al</i> 2008
<i>Foxo3a</i>	x	x	Suppressor	Castrillon <i>et al</i> 2003
<i>Tsc1</i>	x	x	Suppressor	Adhikari <i>et al</i> 2010
<i>Tsc2</i>	x	x	Suppressor	Adhikari <i>et al</i> 2009
<i>p27</i>	x	x	Suppressor	Rajareddy <i>et al</i> 2007
AMH	x	Suppressor	Suppressor	Durlinger <i>et al</i> 1999, Nilsson <i>et al</i> 2011
Kit ligand	x	?	Activator	Nilsson and Skinner 2004
PDGF	x	?	Activator	Nilsson <i>et al</i> 2006
<i>GDF9</i>	x	x	Activator	Dong <i>et al</i> 1996
<i>BMP15</i>	x	x	Activator	Galloway <i>et al</i> 2000

1.1.4 Early folliculogenesis

As the follicle progresses from the primary stage, the granulosa cells continue to proliferate and form multiple layers, the oocyte continues to grow and a thecal cell layer is recruited. The thecal layer becomes heavily vascularized, reflecting the dependence of the preantral follicle on endocrine support. Fluid filled patches then form in the granulosa cell layers, which coalesce to form an antral cavity (Fig 1.1.). The exact function of the antral cavity is unclear but is considered to be a nutrient store as well as potentially aiding in the maintenance of the correct pH and as a sink for oocyte and granulosa cell waste products.

The cells of the follicle are interdependent on each other for the correct growth and development, with the oocyte considered to be the driving force behind overall follicle development (Eppig *et al*, 2002). Bidirectional communication between the oocyte and somatic cells of the follicle is essential and so must be tightly coordinated. The oocyte interacts with the granulosa cells through gap junctions which are channels formed from connexin proteins. The importance of these to oocyte growth and development is highlighted by mice lacking the connexin-37 gene, which fail to exhibit mature Graafian follicles, ovulate, have premature luteinization and do not show meiotic competence (Simon *et al*, 1997). The oocyte produces factors such as GDF-9 (Growth Differentiation Factor-9, McGrath *et al*, 1995) and BMP-15 (Bone Morphogenetic Protein-15, Dube *et al*, 1998), which promote the proliferation and differentiation of the granulosa cells. In turn, the granulosa cells support oocyte growth and regulate its progression through meiosis. The oocyte increases in size, at least in part due to an increase in protein content, as

proteins essential for further development, maturation and preimplantation embryo development are synthesised.

During gamete formation, epigenetic reprogramming occurs in a process including widespread demethylation and histone modifications (Seisenberger *et al*, 2013). This means that sex-specific imprinting needs to be established in the gametes and so another important part of oocyte development is epigenetic reprogramming, with maternal gene imprinting occurring as the oocyte grows (Lucifero *et al*, 2002). This is likely to occur through DNA methylation, with specific gene imprints established throughout primary to antral oocyte growth (Obata and Kono, 2002). This process is essential, as parthenogenetic embryos which express double copies of either the paternal or maternal genome arrest in early development (Surani *et al*, 1984).

1.1.5. Late folliculogenesis

Around the time the antral cavity develops, the oocyte stops growing but continues the process of nuclear maturation, developing the ability to resume meiosis, undergo fertilisation and support embryonic development. This process occurs whilst the oocyte is maintained in meiotic arrest, achieved through the interaction of the oocyte with its surrounding granulosa cells. It is thought that oocyte meiotic maturation is inhibited through a cyclic-AMP (Adenosine Monophosphate) dependant process (Eppig *et al*, 1983), levels of which are regulated by cyclic-GMP (Guanosine Monophosphate) produced by the granulosa cells and passed to the oocyte through gap junctions (Norris *et al*, 2009). High levels of cyclic-AMP suppress MPF (Maturation Promoting Factor, a complex of cyclin B and Cdc-2), maintaining meiotic arrest.

The early antral follicle is also dependant on the presence of follicle stimulating hormone (FSH) from the pituitary gland, which it is increasingly exposed to through the increased vasculature in the thecal cell layer. As well as inducing the proliferation of granulosa cells at this follicle stage, FSH also drives the expression of lutenising hormone (LH) receptors on the granulosa cells which are essential for ovulation and corpus luteum formation (Erickson *et al*, 1979). FSH also plays a key role in steroidogenesis, driving the production of oestradiol-17 β in the granulosa cells. Aromatase, the enzyme crucial for oestradiol synthesis is expressed only in the granulosa cells in mice (Gray *et al*, 1995). To synthesise oestradiol, aromatase requires androgens as a substrate. The granulosa cells do not have the enzymes required for androgen production and so are reliant on androstenedione produced by the surrounding thecal cells. This is known as the 2 cell/2gonadotrophin model of steroidogenesis (Hillier *et al*, 1994).

As the antral cavity forms, the granulosa cells differentiate to become two distinct populations (Telfer *et al*, 1988). Those adjacent to the oocyte differentiate to become the cumulus cells, which remain attached to the oocyte and are ovulated with it. The oocyte retains contact with these cells through gap junctions, allowing for the cumulus cells to supply nutrients that allow for further development as well as keeping the oocyte in meiotic arrest (Eppig and Downs, 1984). Those closest to the follicle wall become the mural granulosa cells, which remain behind following ovulation and regress to form the corpus luteum. The final phase of preovulatory development involves a massive increase in follicle size, due to the increase in the volume of follicular fluid.

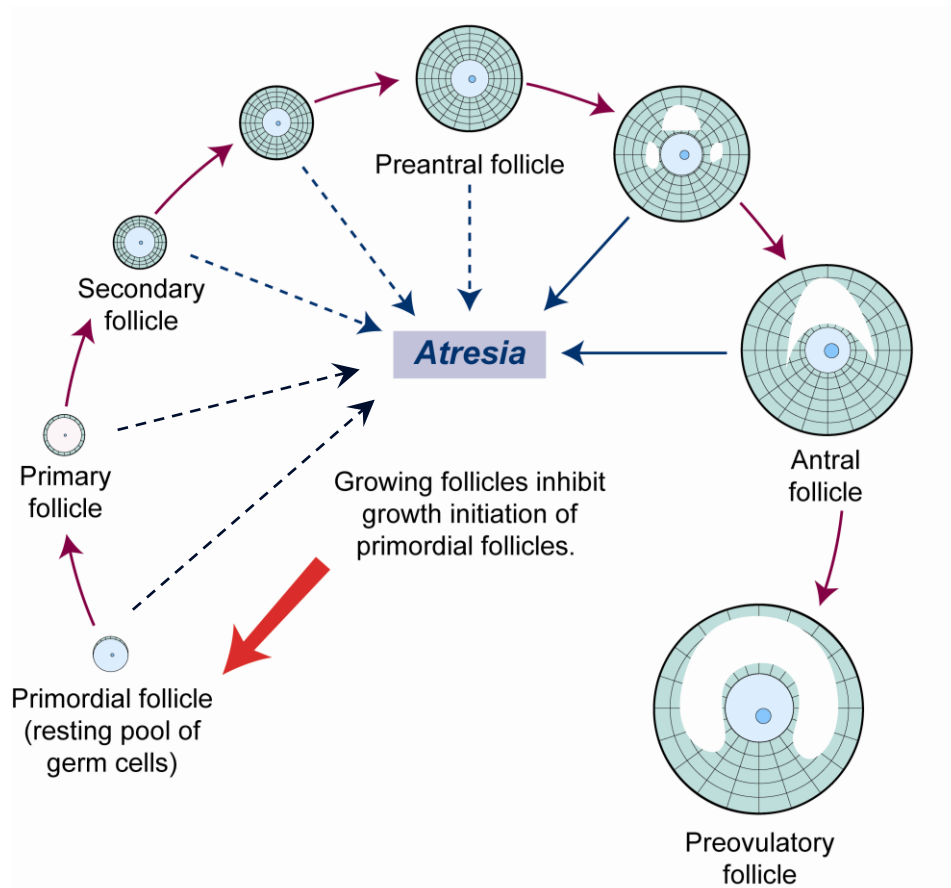


Fig 1.1. Diagrammatic illustration of Folliculogenesis. At any one time, a small cohort of primordial follicles are recruited out of the resting pool and activated to grow. Of the follicles that are activated, only one will make it to the preovulatory stage and be ovulated; the others will die from follicle atresia. Although the majority of follicles which are lost from atresia are from the antral stage (indicated by the solid lines), follicles can be lost from any stage of folliculogenesis (indicated by the dashed lines). Adapted from Morgan *et al*, 2012.

1.1.6. Apoptosis

The ovary is the site of a large amount of cell death physiologically, the majority of which appears to occur through apoptosis. Apoptosis has been shown to have an important role in establishing the primordial germ cell population, follicle atresia as well as corpus luteum regression (evidence summarised in Table 1.2). Apoptosis is a well-defined mechanism of programmed cell death which is characterised by distinct morphological changes including cell shrinkage, membrane blebbing and DNA fragmentation. The canonical apoptosis pathway is an energy-dependant molecular cascade, which can be activated by intrinsic factors or extrinsic stimuli (e.g. Tumour necrosis factor [TNF]). The disruption of mitochondrial function plays a key role, including loss of transmembrane potential and release of cytochrome c into the cytosol. From there, cleavage/activation of caspase-9 allows for the cleavage/activation of other downstream members of the caspase family which subsequently cleave target proteins including PARP-1 (poly[ADP-ribose] polymerase 1) (Fig 1.2.).

<i>Gene</i>	<i>Action</i>	<i>Ovarian Phenotype/Role in the ovary</i>	<i>Reference</i>
<i>Bax</i>	Pro-apoptotic	Increased germ cell numbers (-/-)	Greenfeld <i>et al</i> 2007
<i>Bcl2</i>	Pro-survival	Increased primordial follicle numbers at birth (overexpressed)	Flaws <i>et al</i> 2001
		Protected from normal and chemotherapy induced apoptosis (oocyte specific expression)	Morita <i>et al</i> 1999
<i>Bid</i>	Pro-apoptotic	Involved in granulosa cell apoptosis	Sai <i>et al</i> 2011
<i>Caspase-2</i>	Pro-apoptotic	Involved in radiation-induced oocyte death	Hanoux <i>et al</i> 2007
<i>Caspase-3</i>	Pro-apoptotic	Required for granulosa cell apoptosis	Matikainen <i>et al</i> 2001
		Expressed in corpus luteum	Carambula <i>et al</i> 2002
<i>Caspase-9</i>	Pro-apoptotic	Involved in neonatal oocyte loss	Ene <i>et al</i> 2013
<i>PARP-1</i>	Pro-apoptotic	Cleaved during germ cell nest breakdown	Pepling and Spradling 2001
<i>p53</i>	Pro-apoptotic	Granulosa cell apoptosis	Tilly <i>et al</i> 1995
<i>TAp63</i>	Pro-apoptotic	Oocyte specific death	Gonfloni <i>et al</i> 2009
<i>Fas ligand</i>	Pro-apoptotic	Granulosa cell apoptosis	Yang <i>et al</i> 2012
<i>TNFα</i>	Pro-apoptotic	Can decrease oocyte and primordial follicle number	Marcinkiewicz <i>et al</i> 2002
<i>c-KIT</i>	Pro-survival	Improved oocyte survival	De Felici <i>et al</i> 1999

Table 1.2. Summary of some of the major factors involved in apoptosis and their role in the ovary.

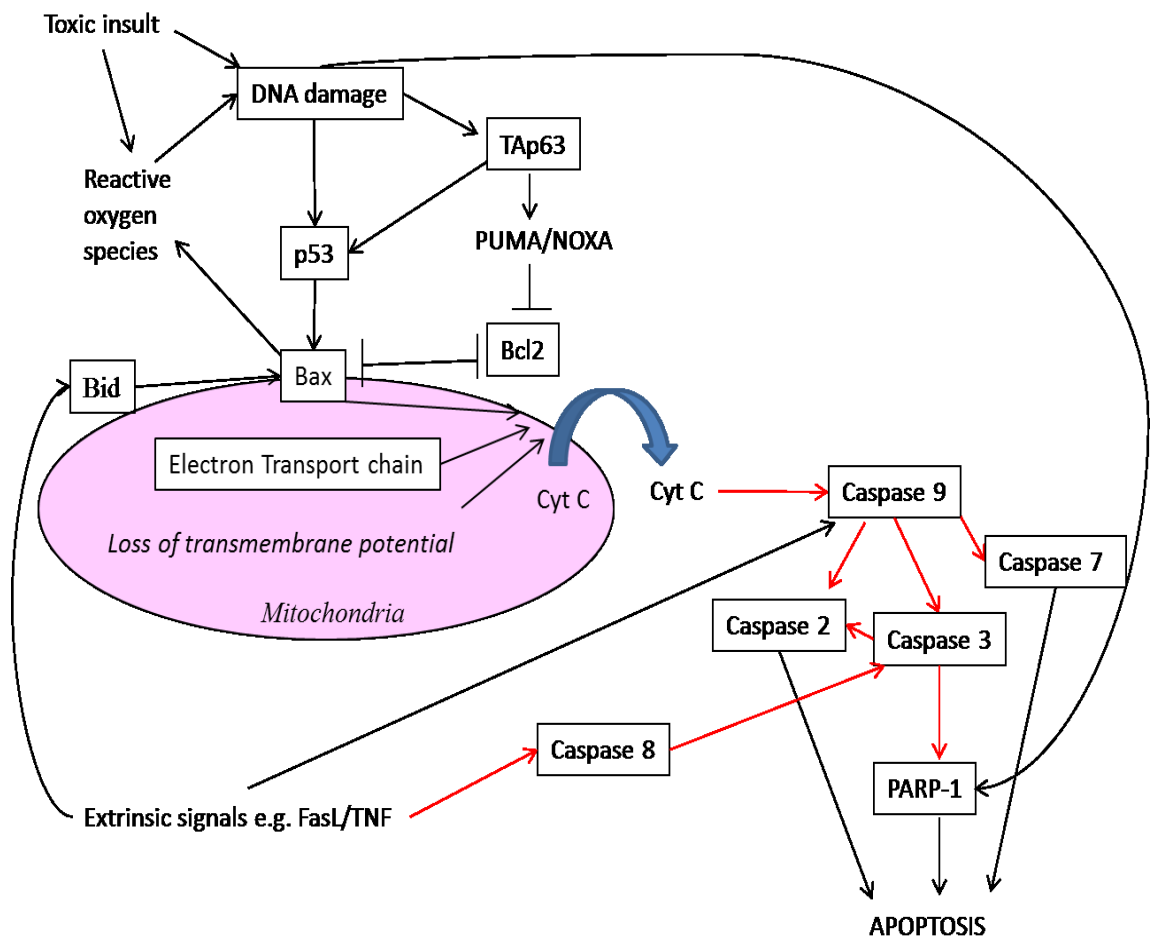


Fig 1.2. Summary of some of the major factors involved in the canonical apoptotic pathway and how they interact with each other. Black arrows indicate activation, flat ended arrows indicate repression and red arrows indicate cleavage/activation.

1.1.7 Ovulation

Ovulation is dependent on a massive surge in LH released from the pituitary gland, receptors for which are expressed on the granulosa and theca cells (Jeppesen *et al*, 2012). Within a few hours of this surge, germinal vesicle breakdown (GVBD) occurs, signalling the resumption of meiosis in the oocyte. This will continue until the first meiotic cell division, an asynchronous division in which half of the chromosomes are ejected from the oocyte but with little cytoplasm (Maro and Verlhac, 2002). This forms the first polar body which sits under the zona pellucida layer. The oocyte continues into the second phase of meiosis until the chromosomes are aligned on the spindle in metaphase (MII oocytes). Meiotic arrest then resumes until fertilisation occurs.

The LH surge induces the expression of genes crucial to ovulation in the granulosa cells of the preovulatory follicle, including progesterone receptors and prostaglandins (Richards *et al*, 1998). Morphologically, the ovary becomes swollen and red which are characteristics of an inflammatory response. Leukocytes and macrophages are recruited to the site as well as factors including cytokines, histamine and prostaglandins (Espey, 1994). At the apex of the follicle, the basement membrane degrades (Reich *et al*, 1985) and so the follicle ruptures, releasing the oocyte with its attached cumulus cells and some follicular fluid adjacent to the fimbriae of the infundibulum of the oviduct. The infundibulum has cilia which help collect the oocyte-cumulus complex and guide it into the oviduct (Hunter, 1998).

The mural granulosa cells which remain behind divide rapidly and then hypertrophy to form large luteal cells which produce increasing quantities of progestagens.

Remaining thecal cells also form luteal cells, which produce progestagens as well as androgens. The whole structure becomes heavily vascularized and is referred to as the corpus luteum.

1.1.8 Fertilisation

The oocyte is conveyed along the oviduct to the ampulla region, a process which is classically attributed to a combination of cilia beating and smooth muscle contractions. Cilia beating is potentially mediated through the progesterone receptor, which is expressed in the cilia of the oviducts epithelial cells (Teilmann *et al*, 2006). The oviduct is lined with smooth muscle, slow wave contractions of which also contribute to oocyte transport (Dixon *et al*, 2011). Once the oocyte arrives in the ampulla, it attaches to the oviductal epithelium through the cumulus cells (Kolle *et al*, 2009)

Spermatozoa enter the female reproductive tract where they capacitate. Capacitation involves the spermatozoa becoming hyperactive, with the flagella beating asymmetrically allowing for progressive motility (Chang and Suarez, 2010). This process also involves changes in membrane organisation and ion permeability (Visconti *et al*, 2011). The changes in the head of the spermatozoa make the acrosome cap unstable, allowing for the acrosome reaction to occur in response to the presence of the oocyte-cumulus cell complex (Zaneveld *et al*, 1991). Capacitation is essential, made clear by early experiments which demonstrated that sperm have to reside in the female reproductive tract before fertilisation can occur (Austin, 1952; Chang, 1951). Sperm bind the oviduct to form a sperm reservoir, seemingly allowing for the release of small quantities of them at a time, possibly as an additional control

to prevent polyspermy. The exact mechanism by which the sperm then detach from the oviduct is unclear but maybe due to their hyperactivity. Once they have detached, they have to recognize the correct direction to travel in order to approach the oocyte-cumulus complex. In mammals this has been attributed to chemotaxis, which is a crucial mechanism in invertebrates (Chang *et al*, 2013) and/or temperature gradients, whereby the sperm move towards increasing temperatures (Bahat *et al*, 2003).

The spermatozoa approach the oocyte cumulus complexes which are in the ampulla region of the fallopian tube and penetrate through the cumulus cell layer to reach the zona. The cumulus cells are embedded in an extracellular matrix rich in hyaluron (Kim *et al*, 2008), therefore the sperm utilise hyaluronidase enzyme which digests a path through the cumulus cells, as well as their own motility. Spermatozoa then bind to the zona pellucida, the exact process of which remains somewhat controversial. In mice, initial experiments indicated that sperm binding is mediated through the ZP3 protein (Bleil and Wassarman, 1983). Recent evidence suggests that in humans, ZP2 is the key to this process as human sperm only binds to zona pellucidae expressing ZP2, either alone or co-expressed with the other ZP (Baibakov *et al*, 2012). It is generally assumed that this binding induces exocytosis of the contents of the acrosomal cavity; also known as the acrosome reaction. Recent evidence has suggested however that in mice, many spermatozoa begin the acrosome reaction before contact with the zona pellucida, suggesting that the cumulus cells may play a role in triggering this reaction (Jin *et al*, 2011). The acrosome reaction is essential for the sperm to penetrate the zona and fuse with the plasma membrane. As it is only acrosome reacted sperm which are capable of fusing to the oocyte membrane, it is

thought that the acrosome reaction allows the release of proteases such as acrosin which aid this binding (Howes and Jones, 2002).

The plasma membrane of the oocyte is then modified to prevent any additional sperm from binding to the egg. This is a rapid process, occurring within minutes, but unlike other species, in humans it does not appear to be associated with changes in electrical potential (Jaffe *et al*, 1983) and the exact mechanism is unclear. In the mouse, fusion of the sperm head with the oocyte causes a calcium-dependant exocytosis of cortical granules, which contain enzymes that modify the plasma membrane (Ducibella *et al*, 1994). A further block to polyspermy in most species occurs as ZP2 is cleaved to prevent more sperm binding (Burkart *et al*, 2012).

The oocyte at this stage is still in meiotic arrest and so must undergo a further cleavage to complete MII. Fertilisation induces the second meiotic division, leading to the extrusion of a second polar body. This occurs due to the increase in intracellular calcium, which inhibits expression of MPF, the factor which maintains meiotic arrest. The paternal genome is decondensed, protamines are removed and the DNA is demethylated (Mayer *et al*, 2000). Shortly after fertilisation, nuclear membranes form around the maternal and paternal chromosomes, which become identifiable as two distinct pronuclei in the centre of the zygote. DNA replication occurs here before the nuclear membranes breakdown and the chromosomes assemble on the mitotic spindle (Bouniol *et al*, 1995). Cell division then occurs and the one cell zygote becomes a two-cell embryo, approximately 24 hours after fertilisation. The embryo at this stage is reliant on RNA and proteins which were present in the oocyte and synthesised during oogenesis. The zygote genome is activated at the late 1-cell to the mid 2-cell stage, allowing the embryo to produce its

own RNA and proteins and so most of the maternal mRNAs remaining at this stage are degraded (Paynton *et al*, 1988)

1.1.9 Preimplantation Development

Preimplantation development of the embryo is defined as the period in which the two cell embryo becomes a multicellular expanded blastocyst and implants into the endometrial lining of the uterus.

Cell cleavages beyond the 2-cell stage occur as the embryo moves along the oviduct, with the embryo comprising 4 and then 8-cells, with individual cells called blastomeres. Following these 3 cell divisions, the embryo compacts to form a morula. The blastomeres become smooth and flattened and cell adhesions increase due to the formation of intracellular junctions meaning that cell boundaries are less distinct (Reima, 1990). The blastomeres also become polarized, with a dense population of microvilli appearing on their apical surface (Johnson and McConnell, 2004). The blastomeres then specify into two distinct lineages. The trophectoderm (TE) forms as the outer epithelial layer of the blastocyst and will develop into the extraembryonic tissue including the placenta (Sasaki, 2010). The inner cells form the inner cell mass (ICM) which develops eccentrically with a fluid filled cavity (blastocoel) forming at the 32 cell stage. The blastocyst then hatches out from the zona pellucida and implants into the uterine endometrium.

The environment of the oviduct and uterus needs to support the development of the zygote through to the blastocyst stage. Development to the blastocyst stage can be achieved *in vitro*, so much of our knowledge of the factors required *in vivo* has been derived from such experiments. An appropriate energy source is essential and whilst

the zygote preferentially metabolises pyruvate, the compacted morula and blastocyst utilise glucose as their main energy source (Hardy *et al*, 1989). Within the female reproductive tract, there is a carbohydrate gradient which reflects the needs of the embryo as it moves through and develops. This is why many *in vitro* fertilisation protocols utilise sequential media which are designed to support different stages of embryo development. Amino acids are also important and their addition to culture improves embryo viability (Gardner *et al*, 2002).

Epigenetic reprogramming is a key event in early embryo development. The paternal genome is actively demethylated in the zygote, with a more passive demethylation of the maternal genome occurring through to the morula stage. At the blastocyst stage, *de novo* methylation of DNA occurs in the inner cell mass (Santos *et al*, 2002).

1.2 *In vitro* culture of ovarian tissue

In vitro culture of ovarian tissue has been a useful experimental tool to elucidate key events and regulators in the process of folliculogenesis. In the mouse, maturation from primordial follicles through to live young has been achieved in culture, indicating that *in vitro* conditions are highly physiological (O'Brien *et al*, 2003). Full maturation from primordial follicles to fertilisable oocytes *in vitro* has not yet been achieved in humans, although great strides forward have been made (Telfer and Zelinski, 2013). The large number of primordial follicles present in ovaries/ovarian biopsies makes them an attractive prospect for fertility preservation, as a system to achieve full maturation *in vitro* could have far reaching benefits for assisted reproduction, particularly for patients following chemotherapy treatment. Culture of

ovarian tissue also provides a useful research tool for toxicology testing of agents including chemotherapy.

There are many challenges involved in the successful culture of ovarian material *in vitro*. Optimisation of the culture medium is necessary if it is to adequately support the physiological needs of each cell type as well as the follicle as a unit. The base medium used varies and includes minimum essential medium (Spears, 1994), McCoy's 5a (Telfer *et al*, 2008) and Waymouth's (Eppig and O'Brien, 1996). The base medium is then often supplemented. Serum is a heavily debated additive, as it has a highly variable composition of substrates including hormones, proteins, carbohydrates and growth factors (Picton *et al*, 2008). Its addition is usually necessary as it acts to balance the osmolarity and can provide substrates for steroid biosynthesis. Serum is often replaced therefore with bovine serum albumin (BSA) or serum substitutes, which can successfully support follicle development (Telfer *et al*, 2008). Other common additives include antibiotics, FSH, insulin, selenium, transferrin, ascorbic acid (Murray *et al*, 2001) and activin (Telfer *et al*, 2008). The differences in additives reflect the different species used and the changing requirements of each stage of follicle development.

Rodent follicle culture is much further advanced than in other species, with such successes difficult to replicate in humans and other large ruminants. It is technically challenging to retrieve follicles from the ovarian cortex of these animals, due to the fibrous stroma (Telfer, 1996). Primordial follicles when isolated degenerate rapidly in culture, indicating that they require the support of other cells of the ovary (Hornick *et al*, 2013). Primordial follicles can therefore be cultured successfully within pieces of ovarian cortex which provides them with the support of their surrounding cells,

maintains the three-dimensional structure of the tissue and can avoid damage from enzymatic retrieval procedures.

For follicles to develop further, they must be isolated from the tissue at around the secondary stage, either mechanically or with enzymatic digestion although the use of enzymes can cause a loss of follicular integrity. Murine preantral follicles can be cultured in microdrops of culture medium overlayed with mineral oil, which support development from the mid-preantral stage to the Graffian stage. These follicles contain oocytes which are healthy and can be fertilised to produce live offspring (Spears *et al*, 1994). Other approaches for preantral culture include encapsulating the follicle in a matrix of collagen or alginate (Carroll *et al*, 1991; Xu *et al*, 2006). The final stage is *in vitro* maturation (IVM) of oocytes, which will support the maturation of oocytes through further meiotic progression to the MII stage. Whilst it still can be considered fairly experimental, IVM using human oocytes has been achieved clinically (Ferraretti *et al*, 2013).

In vitro fertilisation of oocytes once matured is a well-established clinical technique to treat problems with fertility. It is estimated that 1-2% of births worldwide can be attributed to IVF technologies (Fortunato and Tosti, 2011). IVF has also greatly increased our understanding of the key events and factors required during early embryo development.

1.3 Chemotherapy and Premature Ovarian Failure

Advances in chemotherapy treatment are leading to increased survival rates among cancer patients. In premenopausal women, chemotherapy treatment has been linked to premature ovarian failure (POF) due to accelerated follicle loss. As well as its

detrimental effect on fertility, POF has a number of associated side effects including an increased risk of cardiovascular disease (Jeanes *et al*, 2007), osteoporosis (Bruning *et al*, 1990) and psychosocial problems such as depression (Carter *et al*, 2005). Chemotherapy is not the only risk factor for the development of POF in premenopausal women; other major risk factors are summarised below (Table 1.3.).

<i>Genetic</i>	<i>Reference</i>
Fragile X syndrome (e.g. FMR1 mutation carriers)	De Caro <i>et al</i> 2008
Turners syndrome and haploinsufficiency of X-linked genes	Goswami and Conway 2005
Autosomal gene deletion/mutations (e.g. GDF9, FOXL2, FSHR)	Jin <i>et al</i> 2012
Family history of POF	Van Kasteren <i>et al</i> 1999
<i>Other risk factors</i>	
Autoimmune disease	Dragojević-Dikić <i>et al</i> 2010
Metabolic disease (e.g. galactosaemia)	Fridovich-Keil <i>et al</i> 2011
Environmental/lifestyle toxins (e.g. 4-vinylcyclohexane diepoxide and cigarette smoke)	Hoyer <i>et al</i> 2001
Chemotherapy exposure	Morgan <i>et al</i> 2012
Radiotherapy	Wo and Viswanathan 2009

Table 1.3. Major risk factors associated with diminished ovarian reserve and POF.

The risk of developing POF following chemotherapy treatment is dependent on several factors. The chemotherapeutic regimen used is important, with data suggesting that some treatments, such as alkylating agents, are more gonadotoxic than others. Following treatment with alkylating agents, 42% of women developed

premature ovarian failure whilst those exposed to plant alkaloids or platinum agents had no significant increase in POF risk (Meirow, 2000). It has been estimated that 60-80% of women who are treated with CMF (cyclophosphamide, methotrexate and 5-fluouracil) will develop premature ovarian failure (Byrne *et al*, 1992). In women under the age of 20, those treated with alkylating agents were nine times more likely to develop premature ovarian failure than control patients (Bines *et al*, 1996; Lower *et al*, 1999). Dosage of treatment is another important factor as several studies have shown premature menopause to occur in a dose dependant manner (Chiarelli *et al*, 1999). In mice, there was an increase in primordial follicle loss following increasing doses of cyclophosphamide (Meirow *et al*, 1999).

Another crucial risk factor is the age of the patient, with an increased incidence in POF reported in older patients (Petrek *et al*, 2006). Older women have a smaller primordial follicle pool than younger patients at the start of treatment, so it could be that follicle loss from this smaller pool is more likely to induce POF by the end of treatment (Meirow, 2000).

One of the major issues with studies designed to assess the risk of a patient developing POF following chemotherapy treatment, is the reliability and heterogeneity of methods used to assess the remaining follicle pool. One commonly used endpoint is amenorrhea but this can be a temporary effect as patients often resume menstruation in the months following treatment. This is clearly demonstrated in a prospective study which found that, although 84% of women treated with doxorubicin and cyclophosphamide became amenorrhoeic during treatment, almost half had resumed menstruating nine months later (Petrek *et al*, 2006). This resumption can, however, be shortlived before permanent amenorrhoea occurs (Sklar

et al, 2006). Amenorrhea is also an unreliable output measure as it is often self-reported. Other measures which are being used increasingly frequently are ultrasound antral follicle counts and hormonal indicators including oestradiol, FSH, inhibin B and AMH. AMH is produced by the granulosa cells of growing follicles and so serum levels can be used to infer the number of small growing follicles present. From this, the number of remaining primordial follicles can be extrapolated (Kelsey *et al*, 2011).

What is missing from the vast majority of publications are the long-term follow up data which is needed to establish if women who resume menstrual cycles subsequently undergo POF (Bines *et al*, 1996; Letourneau *et al*, 2012; Partridge and Ruddy, 2007). The major difficulty with these studies is that they would need to follow women for decades. To date, this has been done in two studies of childhood cancer survivors. The reproductive outcomes for 830 cancer survivors were analysed in one study to determine the risk of developing POF following either chemotherapy or irradiation (Chiarelli *et al*, 1999). The long term follow up was between 5 and 30 years and showed that there was no increase in risk of POF in chemotherapy treated patients. However the patients who were treated only with chemotherapy were the patients that had the fewest years of follow up (45% had less than 10 years follow up and so were younger than 30 when the study was conducted) and so perhaps any POF may not have become evident yet. Another study, which followed a large number of patients diagnosed between 1970 and 1986, demonstrated that there was a significantly increased risk of developing POF immediately following cyclophosphamide treatment (Sklar *et al*, 2006).

1.3.1 Potential targets in the ovary

1.3.1.1. Follicle class

A woman's reproductive lifespan is determined by the number of primordial follicles present in her ovaries, with the loss of this reserve resulting in POF. This could occur due to a direct effect, with chemotherapy directly targeting the primordial follicle reserve. However, this could also be attributed to an indirect effect, with the death of large activated follicles leading to an increase in primordial follicle recruitment and hence primordial follicle loss (Fig 1.3.A). Growing follicles inhibit primordial follicle activation through factors including AMH (Durlinger *et al*, 1999) and so acute loss of growing follicles could lead to increased recruitment of primordial follicles. Over repeated cycles of chemotherapy treatment, this could lead to a marked effect on the number of primordial follicles remaining in reserve (Morgan *et al*, 2012). In a recent study, cyclophosphamide has been shown to cause increased recruitment of primordial follicles which could occur either through direct action on the primordial follicles themselves, or indirectly through loss of inhibition from growing follicles (Kalich-Philosoph *et al*, 2013).

The follicle population in the ovary is heterogeneous and each follicle class could have a different vulnerability to chemotherapy-induced toxic effects. Currently, there is relatively little data available on this, as most studies examine the primordial follicle reserve, as it is this reserve which determines a woman's reproductive lifespan. When human ovarian biopsies are treated with cyclophosphamide, significantly fewer primordial follicles remain when compared to untreated controls (Oktem and Oktay, 2007b). Following cisplatin and paclitaxel exposure to rats *in*

vivo, primordial follicle counts decrease (Yucebilgin *et al*, 2004). In mouse and rat ovaries, cyclophosphamide and its metabolites cause a decrease in healthy primordial and small primary follicles (Desmeules and Devine, 2006; Petrillo *et al*, 2011).

Damage to the growing population of follicles could lead indirectly to the loss of the primordial follicle reserve. Preantral follicles are highly susceptible to chemotherapy-induced damage, with the quality of follicles deteriorating both *in vivo* and *in vitro* (Abir *et al*, 2008; Raz *et al*, 2002). The rapid fall in AMH levels which is often seen during chemotherapy treatment also indicates that follicles in the preantral/early antral stage are lost (Decanter *et al*, 2010; Rosendahl *et al*, 2010a). The number of secondary follicles present in mouse ovaries significantly reduces following doxorubicin injections (Ben-Aharon *et al*, 2010).

1.3.1.2 Germ or somatic cells

The ovary is populated with several cell types, all of which may be at risk of chemotherapy-induced damage and potentially with differing sensitivities. A common assumption is that the oocyte is damaged directly by chemotherapy treatment, leading to germ cell death and hence follicle loss. There is actually little available evidence for this and so an alternative explanation is that chemotherapy damages the somatic cells of the ovary. The somatic cells are mitotically dividing, a trait which chemotherapy agents are designed to target, and so it could be that chemotherapy causes follicle loss indirectly (Fig 1.3.B). For example, the oocyte and the granulosa cells are interdependent on each other for growth and development and so damage to the granulosa cells would lead indirectly to oocyte damage and hence follicle loss. This could be a latent effect, only manifesting when the granulosa cells

actively divide as the follicle activates. Oocyte damage could also be a latent effect, manifesting at later stages of development including late folliculogenesis or even post fertilisation.

Doxorubicin can cause apoptosis in mature ovulated mouse oocytes (Jurisicova *et al*, 2006; Perez *et al*, 1997) but the effect on immature oocytes contained within follicles is less clear. In an *in vitro* study of human primordial follicles, doxorubicin exposure caused apoptosis and DNA damage in both oocytes and granulosa cells (Soleimani *et al*, 2011). A xenografting model, which grafted human foetal ovary pieces into mice which were then treated with cyclophosphamide, apoptosis was detected in oocytes before pregranulosa cells (Oktem and Oktay, 2007a). Whilst this model could indicate that the oocyte is more susceptible to damage than the granulosa cells, it used foetal tissue which could still contain mitotically active germ cells. When human cortical pieces are cultured in the presence of cyclophosphamide and examined, there is an increase in damaged granulosa cell nuclei and basement membranes after treatment (Raz *et al*, 2002). Similar results were seen in a follow on *in vivo* study, examining ovarian biopsies from chemotherapy treated women (Abir *et al*, 2008). Finally, in mouse ovaries exposed *in vivo* to doxorubicin, DNA damage (as detected by TUNEL positive staining) was first identified in granulosa cells, with expression moving to the oocytes over time (Ben-Aharon *et al*, 2010).

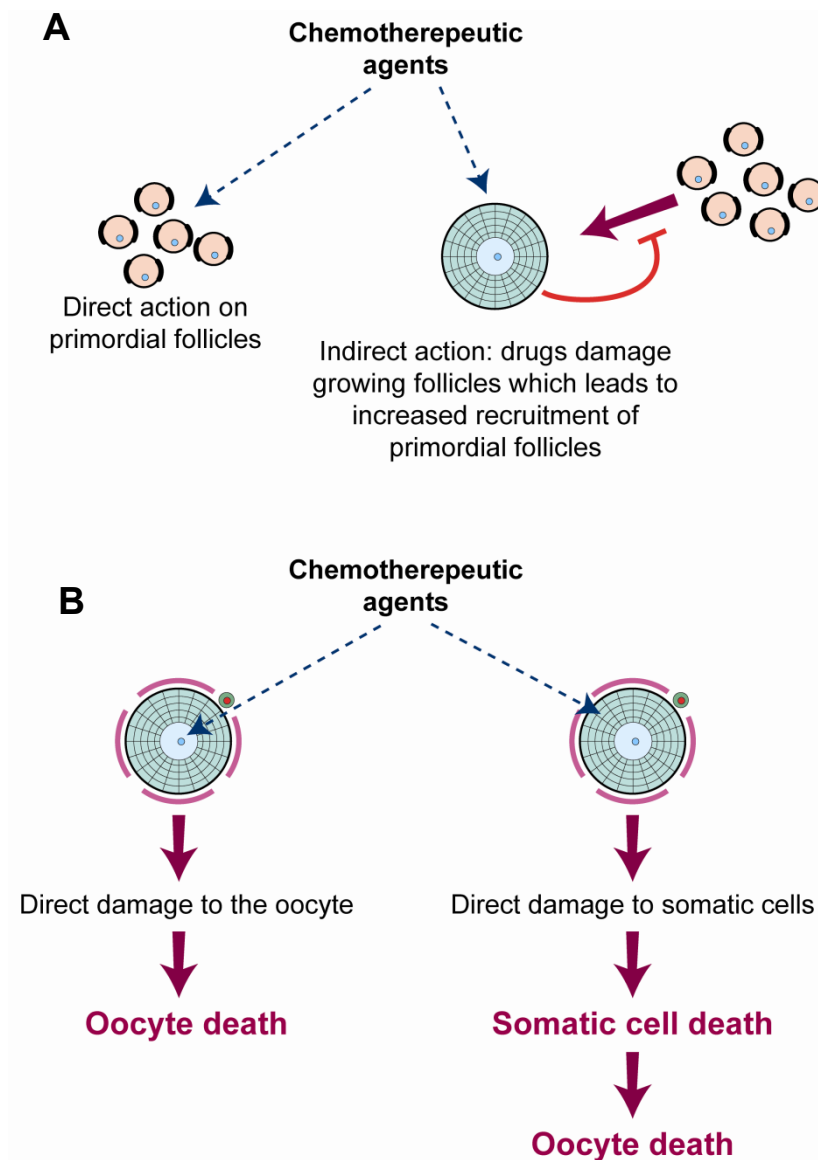


Fig 1.3. Chemotherapy treatment has several potential targets in the ovary. **A)** Chemotherapeutic agents could directly damage the primordial follicle pool or indirectly, through growing follicle damage. As growing follicles inhibit the activation of primordial follicles, loss of the growing population could lead to increased recruitment of primordial follicles and so loss of that reserve. **B)** Chemotherapy could be directly damaging the oocyte or be damaging the somatic cells, which will lead indirectly to oocyte damage and follicle loss. Adapted from (Morgan *et al*, 2012).

Another possibility is that chemotherapy damages the ovarian stroma, which could also adversely affect follicle health. In an ultrastructural study of ovarian biopsies from girls who underwent treatment for childhood leukaemia, moderate to severe signs of stromal damage and capillary changes were found (Marcello *et al*, 1990). Another study has also found evidence of cortical fibrosis and blood vessel damage in human ovaries following chemotherapy (Meirow *et al*, 2007), which suggests that local ischemia may be an additional mechanism by which chemotherapy can cause follicle loss.

Chemotherapy regimen	Author	Model species	Affected cell type	Affected follicle class
Doxorubicin	Perez <i>et al.</i> , (1997)	Mouse	Oocyte	-
	Juriscova <i>et al.</i> , (2006)	Mouse	Oocyte	-
	Ben-Aharon <i>et al.</i> , (2010)	Mouse	Granulosa cells	Secondary
	Bar-Joseph <i>et al.</i> (2010)	Mouse	Oocytes	-
	Soleimani <i>et al.</i> , (2011)	Human, Mouse	Oocyte, Granulosa cells, Stroma, Blood vessels	Primordial, Preantral
Cisplatin	Gonfloni <i>et al.</i> (2009)	Mouse	Oocyte	Primordial, Primary
	Kim <i>et al.</i> (2013)	Mouse	Oocyte	Primordial
Cisplatin/Paclitaxel	Yucebilgin <i>et al.</i> (2004)	Rat	-	Primordial
Irinotecan	Utsunomiya <i>et al.</i> (2008)	Mouse	Granulosa cells	Preantral, Antral, Preovulatory
Cyclophosphamide	Zhao <i>et al.</i> (2010)	Rat	Granulosa cells	-
	Desmeules and Devine (2006)	Mouse	Oocyte and granulosa cells	Primordial, Primary
	Petrillo <i>et al.</i> (2011)	Mouse, Rat	Mainly Oocyte	Primordial, Primary
	Oktem and Oktay (2007)	Human	Oocyte	Primordial
	Raz <i>et al.</i> (2002)	Human	Granulosa cells	-
	Kalich-Philosoph <i>et al.</i> (2013)	Mouse	Granulosa cells	Large growing follicles
Cyclophosphamide, busulfan/carmustine, V, MTX, I-Fos, JET/ Hydroxyurea, imatinib/ HAM, AI, AIE/ Cyclophosphamide, MTX, I-Fos, etoposide, V/MTX, JET/ABVE, CVPP/ABVE/carmustine, V, MTX, E-PIE, JET/adriamycin, etoposide, I-Fos, ACTD, cyclophosphamide, V/ VCAIE/ BEA, CVPP/ABVE, BECOPP/ABVE, CVPP/cyclophosphamide/VMTX/AC/VC/CAF/C, ADR,5FU	Abir <i>et al.</i> (2008)	Human	Granulosa cells, oocyte	Preantral
ADE-GMTZ/CHOP/ Etoposide+ MTX/ ABVD/ CHOP+RT/ Idarubicin cytarabine/ AC/ BEACOPP	Oktem and Oktay (2007)	Human	-	Primordial

Pr,V,DNR,asparaginase,6MP,MTX,COAP/PVD,cyclophosphamide,asparaginase,6MP,MTX/PVD,asparaginase,6MP,MTX/ Pr,V,6MP,MTX/PVD,COAP,6MP,MTX,cyclophosphamide,/Pr,V,daunorubicin,asparaginase,6TG,cytosine arabinoside, doxorubicin, 6MP, MTX/Pr,V,daunorubicin,cytosine arabinoside,6TG,COAP	Marcello <i>et al.</i> (1990)	Human	Stroma, capillary	-
ABVD/MECC/BEACOPP/ VACOP-B/ MINE-EASHAPP/ MTX, V, P/ GMALL/ melphalan, V, nitrogen mustard, gemcytabine, cisplatin, carmustine, etoposide, cytarabine/cytarabine, daunorubicin	Meirow <i>et al.</i> (2007)	Human	Blood vessel	-

Table 1.4. Summary of literature which examines the direct action of chemotherapy on the ovary. ABVD: adriamycin, bleomycin, vinblastin, dacarbazine; ABVE: adriamycin, bleomycin, vinblastin, etoposide; AC, adriamycin, cyclophosphamide; ACTD: adriamycin, cyclophosphamide, actinomycin D; ADE-GMTZ, Arabinoside-C, Daunorubicin, Etoposide, and Gemtuzumab ozogamicin; AI: arabinoside cytoside, Idarubicin; AIE: arabinoside cytosine, Idarubicin, etoposide; BEA: bleomycin, etoposide, adriamycin; BEACOPP; bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, prednisone; BECOPP: bleomycin, etoposide, cyclophosphamide, oncovin, procarbazine, prednisone; CAF: cyclophosphamide, adriamycin, 5-fluorouracil; CHOP, cyclophosphamide, doxorubicin, vincristine, prednisone; COAP: cyclophosphamide, vincristine, cytosine arabinoside, prednisone; COPP: cyclophosphamide, oncovin, procarbazine, prednisone; CVPP: cyclophosphamide, vincristine, procarbazine, prednisone; DNR: Daunorubicin; E-PIE: etoposide, cisplatin, I-fosphamide; 5FU: 5-fluorouracil; GMALL: methotrexate, cyclophosphamide, vincristine, daunorubicine, sparginase, cytarabine, 6-mercapto-purine, etoposide; HAM: high dose arabinoside cytosine, mitoxantrone; I-Fos: I-fosphamide; JET; carboplatin, etoposide; MECC, melphalan, etoposide, citarabin, carmustine; MINE EASHAP, mesna, ifosfamide, mitoxantrone, etoposide, cytosine arabinoside, cis-platinum and steroids; 6MP: 6-mercaptopurine; MTX, methotrexate; P, procarbazine; Pr: prednisone; PVD: Prednisone, vincristine, doxorubicin; 6TG: 6-thioguanine; V, vincristine; VACOP-B, doxorubicin, cyclophosphamide, vincristine, bleomycin, etoposide; VC; vincristine, cyclophosphamide; VCAIE; vincristine, cyclophosphamide, arabinosidecytosine, Idarubicin, etoposide; VMTX: vincristine, methotrexate. Adapted from Morgan *et al* (2012)

1.3.1.3 Non ovarian effects

Chemotherapy could also potentially affect fertility without damaging the ovary by causing damage to the structure of the uterus and its ability to support a foetus to term. Alterations to the uterine vasculature and musculature could lead to an impairment of its function. Data from women treated with abdominal radiation suggests that there are uterine changes, including a shortening of uterine length, an increase in endometrial thickness and decreased blood flow (Critchley, 1999), all of which could compromise implantation and the development of a successful pregnancy. Radiotherapy has also been linked to adverse prenatal outcomes including preterm birth and small gestational weight (Signorello *et al*, 2006). For women treated only with chemotherapy, results are more encouraging. Pregnancy outcome in 44 survivors of acute lymphoblastic leukaemia treated with chemotherapy was described and no adverse effects were reported (Green *et al*, 1989). It may be therefore that chemotherapeutic agents are less detrimental to the uterus than radiotherapy.

1.4 Chemotherapy and male infertility

Chemotherapy treatment has also been linked to male infertility, causing oligospermia and azoospermia in male survivors of childhood Hodgkins lymphoma (van Dorp *et al*, 2012). As with women, the risk of developing fertility problems are related to patient age, dosage of drug used (Strumber *et al*, 2002) and the type of drug (alkylating agents being the most toxic). In the Childhood Cancer Survivor study, survivors were approximately half as likely to sire a pregnancy than sibling controls, with particularly detrimental effects to fertility noted following high doses

of radiotherapy or alkylating agents (Green *et al*, 2010). Eighty percent of male childhood cancer survivors were azoospermic following high dose cisplatin/alkylating treatment; when several drug regimes were compared, 10-50% of survivors were azoospermic (Romerius *et al*, 2011). Drug treatments including cyclophosphamide, ifosamide and busulfan are considered highly toxic to spermatogenesis, with cisplatin and doxorubicin considered medium risk (Dohle, 2010). Age is a somewhat controversial risk factor in males, with some studies showing the testis is susceptible to damage whether exposed pre- or post- pubertally (Wallace *et al*, 2005).

Whilst chemotherapy is often associated with azoospermia during and immediately following treatment, this can be a short term effect with spermatogenesis often recovered. The drug treatments could target somatic cells (Sertoli and Leydig cells) or the germ cells themselves. Different drug treatments appear to target different cell types with most damaging the differentiated spermatogonia (Meistrich *et al*, 1982). There is concern that drug treatment could damage the spermatogonial stem cells, as it is these which are responsible for repopulating the testis with germ cells. Treatments which do not include cyclophosphamide have been shown to be less toxic to the stem cells, with fertility often recovered (Nurmio *et al*, 2009). In a study examining long-term Leydig cell function, there was no negative effect of standard cisplatin exposure although high cumulative doses of chemotherapy were linked to persistent decreased function (Gerl *et al*, 2001).

1.5. Chemotherapeutic Agents

There are many different chemotherapy agents which are classified according to their general drug action. As discussed above, the varying classes of drug each cause differing levels of ovotoxicity. Some of the drugs most commonly used in the treatment of premenopausal women are discussed below. A summary of potential mechanisms of action is presented in Fig 1.4.

1.5.1. Cyclophosphamide

Cyclophosphamide is classified as an alkylating agent and is one of the most widely used chemotherapy drugs. It was first synthesised in 1958 and approved for use as an anticancer agent in 1959 (Emadi *et al*, 2009). A broad range of cancers can be treated with cyclophosphamide, including breast and lymphoid malignancies and it is often used in conjunction with other anticancer drugs. It is also used as an immunosuppressor for blood and marrow transplantations. Its use clinically is limited by its toxicity, with side effects including haematological effects such as neutropenia, cardiotoxicity and haemorrhagic cystitis (Lallana and Fadul, 2011; Zver *et al*, 2007). Cyclophosphamide and other alkylating agents such as busulfan are considered to be the most ovotoxic chemotherapeutic agents (Meirow, 2000).

Cyclophosphamide is an inactive pro-drug and so needs to be cleaved to its active metabolite phosphoramidate mustard. There are other metabolites produced as cyclophosphamide is cleaved but work suggests that phosphoramidate mustard is the most ovotoxic metabolite (Desmeules and Devine, 2006; Plowchalk and Mattison, 1991). Phosphoramidate mustard results in intra-strand and inter-strand crosslinking of DNA which can interfere with cell division. Granulosa cells which have been treated

with cyclophosphamide exhibit mitochondrial effects, including a reduction in mitochondrial transmembrane potential and an accumulation of cytochrome c in the cytosol which can lead to a downstream activation of apoptosis through caspase signalling (Zhao *et al*, 2010). During cell death, a protein called Bax which is largely found in the cytosol, inserts into the outer membrane of the mitochondria. Cyclophosphamide has been shown to cause a large upregulation of Bax expression (Barekati *et al*, 2012) and this could be how the drug mediates the reduction in mitochondria transmembrane potential, leading to apoptosis. Oxidative stress, for example through increased reactive oxygen species, is another mechanism through which cyclophosphamide is thought to cause cell death and has been particularly linked to adverse side effects including cardiotoxicity (Schimmel *et al*, 2004). Mouse ovaries which are exposed to cyclophosphamide metabolites *in vitro* have an induction of H2AX expression (Histone H2AX, a marker of double strand DNA breaks) predominantly in oocytes but also in the granulosa cells following treatment (Petrillo *et al*, 2011).

1.5.2 Cisplatin

Cisplatin, or cis-diamminedichloroplatinum(II), was first identified as an anti-tumour agent in the 1970s and was approved for clinical use in 1978. It is used as a therapy for various cancers including ovarian, testicular, cervical, bladder and lung cancer. Although extensively used, its clinical use has been somewhat limited by side effects including ototoxicity, renal damage and neuropathy. Cisplatin's exact mechanism of action is still unclear but it is classified as an alkylating-like agent, causing DNA crosslinking and adduct formation. The mechanism by which cisplatin enters cells to get to DNA to form adducts is also undefined, although it has been linked to either

passive diffusion or the copper transporter CT1 (Ishida *et al*, 2002), expression of which is abundant in the ovarian stroma (Kuo *et al*, 2001). Its interaction with DNA causes intra- and interstrand crosslinks which distort the structure of the DNA.

The primary target of cisplatin action in cells appears to be the nuclear DNA (Akaboshi *et al*, 1992) with some evidence suggesting an interaction with the phospholipids in the cell membrane (Speelmans *et al*, 1996) as well as interfering with the cytoskeleton (Kopf-Maier and Muhlhausen, 1992). There is also the possibility that it may interact with the mitochondrial DNA, as evidenced by a study in Chinese hamster ovary cells which had a 4 fold increase in mitochondrial DNA adduct formation following cisplatin treatment (Olivero *et al*, 1995).

DNA adducts could have several different effects on cellular function. They could lead to the inhibition of DNA synthesis and thus an inhibition of DNA replication. Cisplatin can also cause inhibition of gene expression (Evans and Gralla, 1992) and an inhibition of transcription. The ability of a cell to process DNA adducts could have a massive effect on the sensitivity of that particular cell to cisplatin induced toxicity. *E. Coli* which are deficient in DNA repair mechanisms are much more sensitive to the toxic effects of cisplatin than wild type strains (Beck and Brubaker, 1973). The mechanism of cisplatin-DNA adduct repair in cells is thought to be mainly through the nucleotide excision repair (NER) pathway. This involves the recognition of DNA damage, excision of the damaged portion of DNA and then repair of the gap by DNA polymerase and ligase enzymes. If there is a failure of the cell to repair the DNA adduct or the DNA damage is too overwhelming, this will lead to cell death. It is thought that the cell death triggered in this manner is largely apoptosis related.

Another mechanism by which cisplatin has been implicated to cause cell damage is the activation of mitochondrial-related death pathways, leading to the release of cytochrome c into the cytosol (Sancho-Martinez *et al*, 2012). This subsequently leads to activation of the caspase family of proteins and hence apoptosis. Cisplatin has also been linked to endoplasmic reticulum stress (Mandic *et al*, 2003), which subsequently activates caspase 12 and thus apoptosis. More recently, cisplatin has been linked to increased oxidative stress in the testis (Rezvanfar *et al*, 2013). Nonetheless, the primary action of cisplatin on cells appears to be DNA damage induction.

1.5.3 Doxorubicin

Doxorubicin (also called Adriamycin) is an anthracycline antibiotic which has been used to treat Hodgkins lymphoma, breast cancer and soft cell sarcomas since the 1960's (Minotti *et al*, 2004). Although one of the most effective anticancer drugs, its use clinically is limited by its detrimental side effects- in particular cardiotoxicity leading to congestive heart failure (Lipshultz *et al*, 2012; Volkova and Russell, 2011). There also is a link to an increase in secondary malignancies in treated patients (Arslan and Uslu, 2013)

The mechanism by which doxorubicin causes cell death remains unclear despite its widespread clinical use, with many different actions being implicated. Doxorubicin enters cells through passive diffusion (Decorti *et al*, 1989; Skovsgaard and Nissen, 1982), a process which is favoured by high intracellular pH (Swietach *et al*, 2012). The major mechanisms of doxorubicin are postulated to involve inhibition of topoisomerase II (Tewey *et al*, 1984), inhibition of DNA synthesis (Schott and

Robert, 1989) as well as DNA intercalation and crosslinking (Chen *et al*, 2012; Cullinane *et al*, 1994). There is evidence that doxorubicin can also cause the generation of free radicals (Bachur *et al*, 1977) and increased oxidative stress (Lahoti *et al*, 2012). Doxorubicin also has direct membrane effects, interacting with both the cells plasma membrane and the mitochondrial membrane (Murphree *et al*, 1981; Praet *et al*, 1984). However, many studies examining doxorubicins action *in vitro* use concentrations that far exceed serum levels; when this is taken into consideration, the major action of doxorubicin appears to be topoisomerase II inhibition (Gewirtz, 1999). Tumour cells which are resistant to anthracyclines have reduced levels or activity of topoisomerase II and thus reduced double strand breaks (Capranico *et al*, 1987; Deffie *et al*, 1989)

Topoisomerase enzymes allow for the unwinding of DNA by severing the phosphate backbone of one (type I) or both (type II) strands of the double helix. There are two topoisomerase II enzymes; II α and II β , both of which are inhibited by doxorubicin. The anticancer activity of doxorubicin has been linked to its inhibition of topoisomerase II α (Press *et al*, 2011). In cardiomyocytes, inhibition of topoisomerase II β appears to be the primary mechanism through which doxorubicin causes cardiotoxicity (Zhang *et al*, 2012). Topoisomerase poisons such as doxorubicin stabilise the DNA-topoisomerase complex and thus impede the resealing of DNA strand breaks once cut. This means doxorubicin effectively blocks transcription and replication. DNA strand breaks are rapidly detected by the cell and if they are too extensive to be repaired, cell death pathways such as apoptosis are activated. That being said, doxorubicin is associated with a high level of cytotoxicity even with low levels of DNA damage (Gewirtz, 1999). The site of the DNA breaks may be of

importance here as there is evidence that anthracyclines cause DNA breaks at specific gene loci, which are different to that of other topoisomerase inhibitors (Capranico *et al*, 1990; Gewirtz, 1999).

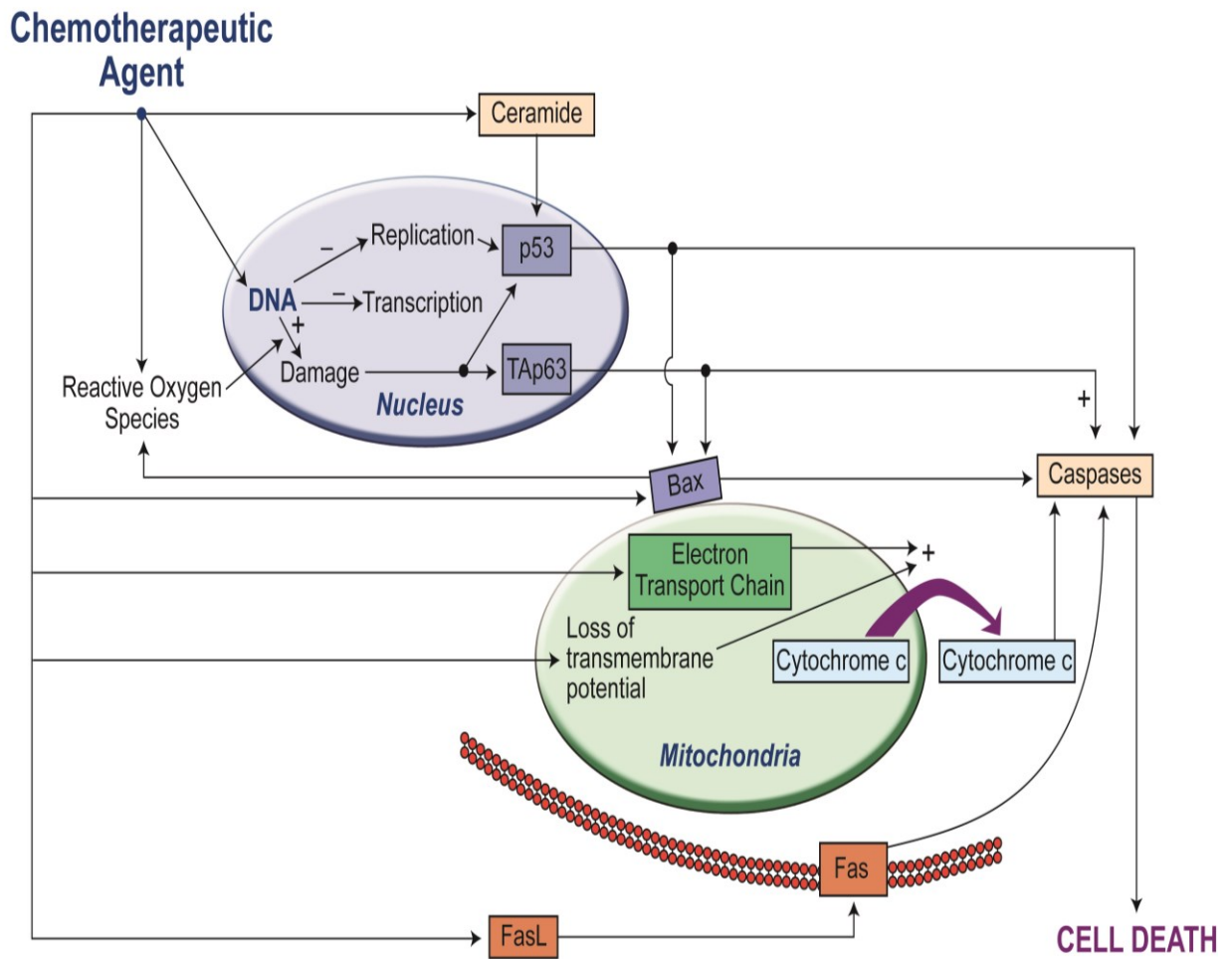


Fig 1.4. Potential mechanisms by which chemotherapy agents may cause cell death. Chemotherapeutic drugs may act at the level of the nucleus to cause DNA damage or interfere with DNA transcription and replication. They can also act on the mitochondria to induce the release of cytochrome c into the cytoplasm. These pathways all interconnect and lead to cell death, often through the caspase family of proteins which are associated with apoptosis. Adapted from Morgan *et al* (2012).

1.6 Hypothesis

Chemotherapeutic agents are designed to target rapidly dividing cells. It is likely therefore that they will damage the somatic cells of the ovary, such as the granulosa cells, thereby causing indirect oocyte loss. The hypothesis of this project therefore was that chemotherapeutic agents cause ovotoxicity primarily through the ovarian somatic cells.

1.7 Aim of this PhD.

With survival rates for cancer patients increasing, more attention needs to be focused on the long term adverse effects of their treatment. Whilst the endpoint of POF following chemotherapy has been well established, the precise mechanism by which this occurs is less clear. Increasing our understanding of the mechanism by which the ovarian follicle pool is depleted could lead to new approaches in treatment to alleviate POF.

The aims of this project were to elucidate in detail the mechanism by which chemotherapeutic agents directly damage the ovary and to determine which stages of follicle development are most affected by chemotherapeutic treatment.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Newborn Mouse Ovary Dissection

2.1.1 Dissection medium

The medium used for dissection in all the methods detailed below was Leibowitz L15 (Invitrogen UK, #11415049). Aliquots of 50mls were stored at 4°C, used within 6 days once opened and then discarded. The osmolarity of each bottle was measured using an osmometer (Camlab, 026461 DBS) and adjusted to 285 mmOsm/kgH₂O using sterile H₂O (Sigma Aldrich, #W1515). This medium was supplemented with 3mg/ml bovine serum albumin (BSA, #A9418, Sigma Aldrich Ltd) under a laminar flow hood and once BSA was dissolved, it was syringe filtered (Filters: Iwaki, #2053-025, pore size 0.2µm, Syringes: BD, #301604). It was warmed in an oven at 37°C for a minimum of 45 minutes prior to use. Once made up, medium was used within 24 hours.

2.1.2 Gross Dissection

Mice were kept on a 12 hour light/dark cycle with food and water available *ad libitum*. Newborn female mice were culled by decapitation (according to UK Home Office Regulations, Project Licence 60/4026) and ovaries were dissected out into glass embryo dishes containing 1ml of Leibovitz L-15 dissection medium supplemented with 3mg/ml BSA (#A9418, Sigma Aldrich Ltd). All dissections were done under a laminar flow hood.

2.1.3 Fine Dissection

Any extra tissue surrounding the ovaries (such as the fallopian tube) was removed using insulin needles (BD Microfine Insulin Needles, 1ml U-100 29G, #037-7606) under a light microscope on a heated stage (heated to 37°C) and the bursa was removed. This was done in a laminar flow hood to ensure sterility.

2.2 Newborn Mouse Ovary Culture

2.2.1 Newborn ovary culture medium

Alpha-MEM medium (Invitrogen, #22571/020) was used for all newborn ovary cultures. Aliquots of 50mls were stored at 4°C and once opened, were used within 6 days and then discarded. The osmolarity of each bottle was measured using an osmometer (Camlab, 026461 DBS) and adjusted to 285 mmOsm/kgH₂O using sterile H₂O (Sigma Aldrich Ltd, #W1515,). In a laminar flow hood, 3mg/ml BSA (A3311, Sigma Aldrich Ltd; this BSA was used as it is more refined than the one present in the L15) was added to the medium. The quantity of medium made up was determined as 1ml of media/ovary with 1ml extra added in case of loss during filtering. Once BSA was added and dissolved, the media was syringe filtered into sterile tubes (Greiner BioOne, #120190). 1ml of medium was pipetted into each well in a 24 well plate (Greiner BioOne, #662160) and a Whatman Nucleopore polycarbonate membrane (#WN/110414, Camlab) was placed on top of each well shiny side up using a pair of sterile watchmaker forceps. Plates were then incubated

in a humidified incubator at 37°C and 5% CO₂ in air for a minimum of 1 hour prior to use.

2.2.2 Newborn ovary culture

Each ovary, once finely dissected, was transferred using a glass pipette onto a Whatman Nucleopore membrane which was floating on top of 1ml alpha-MEM medium (Invitrogen) supplemented with 3mg/ml BSA (Sigma Aldrich) in a 24 well plate (Greiner Bio-one, #662160). Ovaries were randomly allocated into treatment groups. Culture plates were incubated in a humidified incubator at 5% CO₂ in air and 37°C for up to six days. 50% of medium was replaced every 48 hours.

2.3 Chemotherapeutic agent stock solutions

2.3.1 Cisplatin

Cisplatin (cis-Diammineplatinum(II) dichloride, #P4394-25MG, Sigma Aldrich Ltd) was made up in sterile water (Sigma Aldrich Ltd, #W1515) in a class II fume hood to the concentration of 1mg/ml and pipetted vigorously until dissolved. Serial dilutions in sterile water were set up to produce a range of stock solutions including 500µg/ml and 100µg/ml. These were stored in eppendorfs which were parafilmed and wrapped in foil due to light sensitivity. These aliquots were kept at 4°C for up to 6 weeks.

2.3.2 Doxorubicin

Doxorubicin hydrochloride (D1515-10MG, Sigma Aldrich Ltd) was made up in sterile water (W1515, Sigma Aldrich Ltd) in a class II fume hood to the

concentration of 1mg/ml. Serial dilutions in sterile water were set up to produce a range of stock solutions including 100µg/ml, 50µg/ml and 10µg/ml. These were stored in eppendorfs which were parafilmed and wrapped in foil due to light sensitivity. These aliquots were kept at 4°C for up to 4 weeks.

2.4 Histology

2.4.1 Fixing and Processing

Ovaries were removed from membranes using a glass pipette and washed in ice cold PBS (phosphate buffered saline) in a 24 well plate. They were fixed in Bouins or 10% Formalin (Sigma Aldrich, #HT5014) for 75-90 minutes in a class II fume hood. Ovaries were then washed several times in 70% (v/v) ethanol and left overnight in 70% (v/v) ethanol with eosin (approximately 1%) to stain. Ovaries were processed by machine (Sakura Tissue-Tek® VIP, Sakura Fintek UK Ltd, Thatchem UK) and embedded in paraffin. Ovaries were sectioned on a microtome (Leica UK) at 5µm thickness and mounted on poly-lysine coated glass slides (ThermoScientific, #12-545-78).

2.4.2 Haemotoxylin and eosin staining

Sections were dewaxed in xylene for 20 minutes and rehydrated through decreasing concentrations of ethanol (100%, 95%, 90% and 70%) before being washed in running water. Slides were dipped in haemotoxylin (Thermoscientific, #6765004) for approximately 3 minutes before being washed in running water. Slides were acidified in acid alcohol (0.5% [v/v] HCl in 70% [v/v] ethanol) then washed in tap water before being placed in Scotch Tap Water Substitute (STWS, 0.2% NaHCO₃, 2%

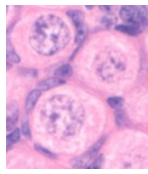
MgSO₄ in dH₂O) for 3 minutes. After another wash in tap water, slides were dipped in eosin (Sigma Aldrich, #E4382, 1% [w/v] solution) for 2 minutes and fixed in potassium aluminate for 2 minutes. After a further wash in tap water, slides were dehydrated through increasing concentrations of ethanol (70%, 90%, 95% and 100%) and placed in xylene to clear for 5 minutes. Slides were then mounted with glass coverslips using DPX (B&D) as a mounting solution.

2.5 Follicle Counting

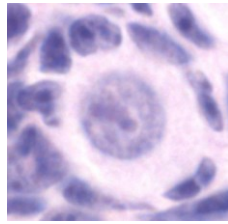
Slides were photographed using a Leica microscope (Leica DMBL with Leica DFC480 camera, Leica UK). For counting, the sixth section of each ovary was photographed to prevent double counting. Images were analysed using Image J software and blind as to treatment group (with the ovaries assigned code numbers at fixation which were decrypted during statistical analysis). A follicle was counted if a germinal vesicle was present in the oocyte. A follicle was considered to be primordial if it consisted of an oocyte surrounded by one or a few flattened pregranulosa cells. A follicle was considered to be transitional if one or more of the surrounding granulosa cells were cuboidal. If all the surrounding granulosa cells were cuboidal and formed one complete layer around the oocyte, the follicle was considered to be primary. If there were two or more complete layers of cuboidal granulosa cells surrounding the oocyte, the follicle was considered to be secondary (Morgan *et al*, 2013).

A follicle was considered healthy if: a) the oocyte was round and contained evenly stained cytoplasm; b) there were no pyknotic granulosa cells (or no more than 1 for primary follicles); and c) there was clear attachment between the oocyte and its

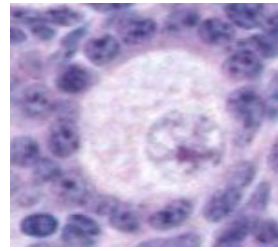
surrounding granulosa cells. Any follicle not considered healthy (not passing all 3 criteria) was further categorized as having an unhealthy oocyte only; unhealthy granulosa cells only; or having both oocyte and granulosa cells unhealthy. An oocyte was considered to be of poor health if it exhibited any one of shrunken cytoplasm, heavy or uneven eosin staining or no attachment between oocyte and its surrounding somatic cells (Fig 2.1). A granulosa cell was considered to be pyknotic if it was shrunken and condensed. The assessment of the health of granulosa cells was dependent on follicle stage: for primordial and transitional follicles, a follicle was assessed as unhealthy if it contained any clearly pyknotic granulosa cell (out of the 3-6 present); for primary follicles, a follicle was assessed as unhealthy if it contained 2 or 3 or more clearly pyknotic granulosa cells (out of the 10-20 present). This criteria was chosen as, in control ovaries, it is rare to see any pyknotic granulosa cells in follicles of these stages. (Morgan *et al*, 2013).



Primordial



Transitional



Primary

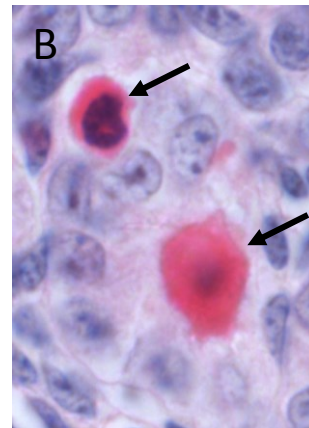
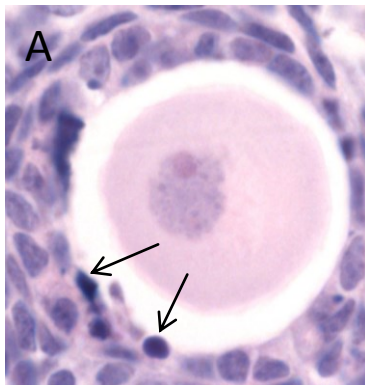


Fig 2.1. Examples of follicles determined as primordial, transitional and primary based on their morphology. Follicles were considered primordial if they consisted of an oocyte surrounded by one or a few flattened pregranulosa cells. Follicles were considered transitional if one or more of the surrounding granulosa cells were cuboidal. If all the surrounding granulosa cells were cuboidal and formed one complete layer around the oocyte, the follicle was considered to be primary. **(A)** A follicle with pyknotic granulosa cells (shown with black arrows). **(B)** Oocytes with shrunken cytoplasm and heavy eosin staining (shown with black arrows).

2.6. Statistical Analysis

For histological analysis, all the follicles in every sixth section of each ovary were counted and classified as described above. Ovaries were assessed with the counter blind as to treatment group, with the ovaries assigned code numbers at fixation which were decrypted during statistical analysis. The sum of the follicles in each category was multiplied by 6 to produce total numbers for each ovary analysed. The average of the total numbers was then quantified for all the ovaries in each treatment group. Graphpad prism software (Graphpad Software Inc, California USA) was used for all data analysis. One way ANOVAs determined if significant differences were present across treatments, followed by Bonferroni post hoc tests where ANOVA was statistically significant. Post hoc tests determined the effect of treatment relative to control.

CHAPTER 3.

THE EFFECT OF CISPLATIN AND DOXORUBICIN ON

THE OVARIAN FOLLICLE POPULATION

3.1 Introduction

3.1.1 Known effects of cisplatin on follicles

Cisplatin acts primarily through causing inter- and intra-strand DNA crosslinking, leading to adduct formation which can interfere with transcription and replication. These adducts, if not repaired appropriately by the cell, can lead to the activation of cell transcription pathways which culminate in the initiation of apoptosis (Siddik, 2003). The effect of cisplatin on follicles within the ovary has only very recently been elucidated, with studies indicating that immature oocytes within follicles are highly susceptible to damage. In neonatal mouse ovaries, cisplatin administration leads to an upregulation of Abl (Abelson murine leukemia viral oncogene homolog 1, Gonfloni *et al*, 2009), a non-receptor tyrosine kinase which is thought to act as a sensor of DNA damage. This in turn leads to an upregulation of TAp63 (Tumour protein p63), a member of the p53 family of proteins which is only expressed in the oocyte. The p53 family act as DNA repair proteins, which can initiate apoptosis if the level of DNA damage present is too high to be repaired (Smith and Suresh Kumar, 2010). Cisplatin treatment causes accumulation of Abl and TAp63 leading to extensive oocyte death (Gonfloni *et al*, 2009). The evidence from the literature to date is that the follicle class that is particularly susceptible to cisplatin within these experimental conditions appears to be the primordial follicle population (Kim *et al*, 2013). The effect of cisplatin on more mature follicle classes is not known.

3.1.2 Known effects of doxorubicin on follicles

There are relatively few studies investigating the action of doxorubicin in the ovary, with the pharmacological mechanisms of action primarily being investigated in other

somatic cell populations; particularly in cardiomyocytes. In somatic cells in general, doxorubicin can interact with mitochondria (Pointon *et al*, 2010), leading to the release of cytochrome c into the cytosol. This in turn leads to activation of apoptotic cascades, including cleavage of the caspase family of proteins. There is also evidence that in the nucleus, doxorubicin can lead to an upregulation of p53 and hence apoptosis. The major problem with applying what has been identified in other studies to the ovary is that most of these studies use very high pharmacological doses of doxorubicin in primary cell lines, the results of which may not be therefore that applicable to serum level doses in whole tissue. Doxorubicin causes apoptosis in mature ovulated oocytes *in vitro* (Jurisicova *et al*, 2006; Perez *et al*, 1997); the effect on more immature oocytes maintained in follicles is less clear. It is also unclear if the drug is more toxic to oocytes or somatic cells when the whole follicle unit is exposed. An *in vitro* study using human ovary cortical strips identified DNA damage and apoptotic cell death in both the oocyte and granulosa cells (Soleimani *et al*, 2011). However this study used much higher doses of doxorubicin (1-100µg/ml) than serum levels in patients. Preliminary experiments in this lab indicated that the primary cell type affected in neonatal mouse ovaries is the granulosa cells (George Liperis, Presented at SRF 2009).

3.1.3 The mouse ovary as an *in vitro* model

The sequence of events underlying follicle formation and folliculogenesis in humans has proven difficult to elucidate experimentally. Human ovarian tissue can be cultured but its use as a research model is more limited due to the scarcity of available material, ovarian size and ethical concerns regarding human embryo

production. Therefore, mouse ovaries have been extensively used as an alternative culture model to examine the earliest stages of folliculogenesis *in vitro*. One advantage of using such a model is that the environment can be tightly controlled, allowing for the elucidation of key regulators and events during this process. Development *in vitro* from the primordial follicle stage through to fertilizable oocytes and live young has been achieved in the mouse (Eppig and O'Brien, 1996; O'Brien *et al*, 2003), indicating that *in vitro* culture can support oocyte development in a physiological manner. Although the mouse ovary model is very useful, there are some drawbacks. Mice are polyovular and have oestrous cycles of 4 days, in contrast to humans who are monoovular and have menstrual cycles of approximately 28 days. There is also comparatively little stromal tissue in the mouse ovary when compared to humans.

The mouse ovary culture system used in the work detailed in this chapter supports follicle formation, growth initiation and development to the primary-secondary phase (Spears *et al*, 2003). In the first 3 days of culture, the predominant follicle class present is primordial. Over the six days most of these will activate so by the end of culture, the majority of follicles are transitional with around 30% classed as primary (Work carried out by Federica Lopes, Fig S1 in Morgan *et al*, 2013, Fig 3.1). As the population of follicles within this culture system is relatively homogenous with only the primordial or early growing classes present, this culture system will allow for determination of the effect of cisplatin and doxorubicin more precisely on the follicle reserve. Another advantage of using this culture system is that the whole ovary can be cultured intact, maintaining the three-dimensional structure of the tissue.

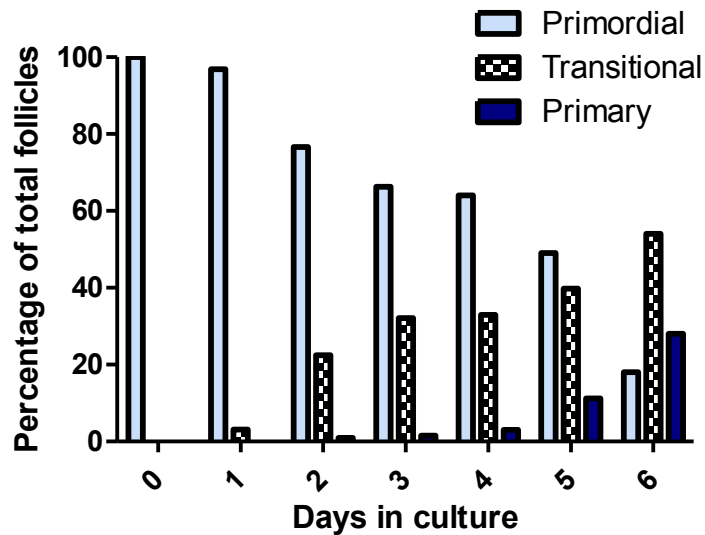


Figure 3.1. The change in follicle distribution over a 6 day culture of newborn mouse ovaries. Work carried out by Federica Lopes, Fig S1 in Morgan *et al*, 2013.

3.1.4. The bovine ovary as an *in vitro* model

Other species are also utilised as models for human ovarian development including rat, sheep, pig, cow and baboon. In large mammalian species such as the cow, *in vitro* culture is more challenging due to the large size of the tissue and the increased density of stromal tissue. The time taken for a primordial follicle to mature to the preantral stage in the cow takes weeks or months *in vivo*, a process which takes only days in the mouse. The process of folliculogenesis in humans and large mammals however is accelerated *in vitro* so that which takes several months *in vivo* takes days *in vitro* (Telfer 1998). The impact of this accelerated development on follicles in terms of their health and their capacity to produce live offspring is presently unclear. The size of follicles is also species dependent, meaning that the culture of bovine antral follicles is more challenging than in the mouse as they are much larger. As

most studies determining appropriate culture conditions for folliculogenesis were conducted initially in the mouse, it has proven difficult to apply these to the culture of bovine tissue, due to the differences in tissue and follicle size and the timing of follicle development. However, there is the argument that bovine tissue may be more appropriate as a model for the human ovary than the mouse. This is because the cow is more similar to humans in terms of being monovular, having folliculogenesis occur over a long time span and having denser stromal tissue.

As it is not possible to culture whole ovaries from the cow as it is in the mouse, a different approach must be taken *in vitro*. If primordial and early growing follicles are isolated and placed into culture, they degenerate rapidly indicating they require the support of stromal cells. Therefore, culturing strips of ovarian cortex maintains this support (Hovatta *et al*, 1999), allowing widespread primordial follicle activation (Wandji *et al*, 1996) and development to the preantral stage. Further development can occur following mechanical isolation of follicles from cortical strips and individual culture thereafter (Telfer *et al*, 2008). These cultures appear to support the follicles fairly well, although no live offspring has been produced yet following culture in the cow, or indeed in any species other than the mouse.

3.1.5 Aims

The aim of the work described here was to determine the effect of cisplatin and doxorubicin on early ovarian follicles. Preliminary experiments to ascertain suitable doses for cisplatin were conducted in both newborn mouse ovaries and bovine cortical strips and results compared to examine any species-related differences.

3.2 Materials and methods

3.2.1 Animals

For the preliminary murine experiments, the mice used were the offspring from C57BL6 X CBA. This was changed to C57BL6 for subsequent experiments comparing the effect of cisplatin and doxorubicin due to low litter numbers available from the crosses. For the bovine cultures, ovaries were obtained from a local abattoir.

3.2.2. Experiment 3.1: Preliminary murine experiments with cisplatin

Newborn mouse ovary culture

Newborn mouse ovaries were dissected and cultured as described in Chapter 2. Medium was supplemented with cisplatin at the final concentrations 0, 0.05, 0.1 and 0.2µg/ml for the full six days of culture. These doses were selected as preliminary experiments exposing individual preantral follicles in culture suggested that they were the lowest concentrations to show an effect (Cultures done by S Morgan). Four ovaries were analysed in each treatment group taken from two independent cultures.

3.2.3. Experiment 3.2: Preliminary bovine cultures with cisplatin

Bovine cortical strip culture

Ovaries were obtained from a local abattoir and transported in Transporting Medium M199 (Hepes buffered, Invitrogen, UK) supplemented with pyruvic acid (25µg/ml), penicillin G (75µg/ml), streptomycin (50µg/ml) and amphotericin B (2.5µg/ml) (all chemicals from Sigma Aldrich Ltd, UK) at 30-35°C, then sprayed with 70% ethanol to avoid contamination. In a laminar flow hood, cortical strips of approximately 1mm

thick were dissected from quiescent appearing areas of cortex i.e. areas containing no prominent follicles or corpus lutea. These were dissected using a scalpel and placed in dissection media [Leibovitz L15 medium (Invitrogen, UK) supplemented with sodium pyruvate (2mM), glutamine (2mM), BSA (#A3912, 3mg/ml), penicillin G (75µg/ml) and streptomycin (50µg/ml)]. 24 well flat-bottomed culture plates were set up with 300µl of control medium [McCoy's 5a medium with bicarbonate supplemented with HEPES (20mM), BSA (A3912, 0.1% [w/v]), glutamine (3mM), penicillin G (0.1mg/ml), streptomycin (0.1mg/ml), transferrin (2.5µg/ml), selenium (4ng/ml), insulin (10ng/ml) and ascorbic acid (50µg/ml), all obtained from Sigma Aldrich Ltd, UK by Marie McLaughlin] in each well. Medium was also supplemented with cisplatin to produce final concentrations of 0, 0.1, 0.2 and 0.4µg/ml. 150µl of medium was removed and exchanged with fresh medium every 48 hours. After 6 days, strips were removed from culture and fixed in Bouins solution. Culture and dissection method taken from Telfer *et al* (2008). Twelve strips were analysed in each treatment group from two independent cultures.

3.2.4. Experiment 3.3: The effect of cisplatin and doxorubicin

Newborn mouse ovaries were dissected and cultured as described in chapter 2. The timing of drug exposure was modified to 24 hours on the second day of culture only. This shorter exposure time was chosen as it more closely models the drug exposure in patients. Following drug treatment, ovaries were transferred into a fresh plate with new membranes and control medium for the final 4 days of culture. The range of concentrations of cisplatin used here were: 0, 0.1, 0.5, 1 and 5µg/ml, whilst those of doxorubicin were: 0, 0.01, 0.05, 0.1 and 0.2 µg/ml. These drug concentrations were

increased from those used in previous experiments as the exposure window is shorter in this paradigm. Five ovaries were analysed in each treatment group with three independent cultures conducted for each drug.

3.2.5 Histology

Following culture, newborn mouse ovaries were fixed in Bouins fixative, processed and embedded in paraffin as described in Experiment 1. Ovaries were sectioned on a microtome at 5µm, stained with haematoxylin and eosin and every 6th section was photographed and counted. Follicles were assessed morphologically as described in Chapter 2 blind as to treatment. Follicle counts were corrected using the Abercrombie correction factor (Abercrombie, 1946). This correction factor takes into account the size of the average germinal vesicle and the thickness of the sections to correct for double counting. Oocyte diameters were measured using Image J software.

Bovine cortical strips were fixed in Bouins for 24 hours, processed through increasing concentrations of ethanol and then placed in cedarwood oil to clear for 24hours. This was removed with toluene, following which cortical strips were embedded in molten paraffin which was exchanged every hour for 3 hours to remove any remaining toluene before cooling. Strips were then sectioned on a microtome at 5µm, mounted on poly-lysine coated slides and stained with haemotoxilyn and eosin as described in chapter 2. Every 12th section was examined under a light microscope (chosen due to advice from Marie McLaughlin) and follicles were counted and

classified blind as to treatment. Follicles counted were expressed as percentages per cortical strip. Follicle health was classified as described in chapter 2.

3.2.6 Statistical analysis

All data were analysed using Graphpad Prism (GraphPad Software, Inc, California USA). Where results were not normally distributed (as determined through Kolmogorov-Smirnoff tests for normality), log transformation was used to normalise. This was used to correct total follicle numbers in Experiment 3.3. One way ANOVA determined if significant differences were present across treatments, followed by Bonferroni post hoc tests where ANOVA was statistically significant. Post hoc tests determined the effect of treatment relative to its control.

3.3 Results

3.3.1 Comparison of mouse and cow as model species

3.3.1.1 Experiment 3.1: Preliminary Murine Experiments with Cisplatin

Newborn mouse ovaries were exposed to 0, 0.05, 0.1 and 0.2 $\mu\text{g/ml}$ cisplatin for 6 days *in vitro* and analysed histologically. These concentrations were chosen since prior experiments culturing individual preantral follicles suggested that they were the lowest concentrations to show an effect. The percentage of follicles classified as unhealthy was significantly higher following 0.1 $\mu\text{g/ml}$ cisplatin (Figure 3.2., $p < 0.001$). At the highest dose, there was an increase in the percentage of follicles classified as unhealthy but this was not significant. This could be due to the low n number of ovaries in this treatment group. When follicle class was analysed, there was an increase in the number of transitional follicles classified as unhealthy in both 0.1 and 0.2 $\mu\text{g/ml}$ cisplatin groups but this was not significant (Fig3.3A). Of the 979 follicles which were classified as primordial, only 7 were classified as morphologically unhealthy and so these data are not shown in the figure. The only follicle type with a significant increase in the percentage classified as morphologically unhealthy were the primary follicles, which increased significantly following 0.1 and 0.2 $\mu\text{g/ml}$ cisplatin (Fig 3.3.B, $p < 0.01$). As this class showed a significant increase in the percentage of morphologically unhealthy follicles, they were further analysed to determine which cell type was affected. The main reason that follicles were classified as unhealthy following cisplatin was due to the oocytes (Fig3.3.C).

3.3.1.2 Experiment 2: Preliminary Bovine Cultures with Cisplatin

Bovine cortical strips were cultured in the presence of cisplatin and assessed morphologically to investigate if there was a similar effect to that observed in the mouse model described above. The concentration range was slightly increased due to the fact that there is denser stroma in bovine tissue which could affect the penetration of the drug into the tissue. Representative histological pictures are shown in figure 3.4. The percentage of follicles classified as unhealthy increased significantly in all doses of cisplatin used (Figure 3.5.A, $p < 0.01$).

Virtually all follicles that were classified as unhealthy were classed as such due to morphologically unhealthy oocytes (of the 5996 total follicles counted, only 66 had unhealthy granulosa cells). When the distribution of follicle classes present in bovine cortical strips was compared to newborn mouse ovaries, there were more significantly fewer primordial and transitional follicles and a significantly higher percentage of primary follicles at the end of culture (Fig 3.6, $p < 0.05$ except for primary follicles where $p < 0.01$).

As both the bovine and mouse cultures show similar results following cisplatin exposure and the mouse has a higher proportion of primordial follicles remaining at the end of culture, all subsequent experiments were conducted using the mouse model. This model was modified for the next experiments to shorten the exposure time of the drug used to 24 hours on the second day of culture only. This was to more closely model clinical conditions, where patients are exposed to a high dose bolus injection of the drug usually on an intermittent basis (Ansell, 2012).

3.3.2. Experiment 3: The effect of cisplatin and doxorubicin

The effect of cisplatin noted in Experiments 3.1 and 3.2 was in contrast to previous experiments conducted in the lab examining the effect of doxorubicin, which showed morphological effects primarily in the granulosa cells of follicles (Murray and Spears, unpublished). Therefore, for the rest of the experiments here and in all subsequent experiments, cisplatin and doxorubicin were used in the same culture system and results compared. The concentration of cisplatin used was increased from the above experiments as the exposure time in this experiment was shorter. The concentrations of doxorubicin used were determined from previous preliminary dose response experiments in the lab (George Liperis, unpublished data). Both drugs were used in doses within the therapeutic range for patients, which are around $0.5\text{--}1\ \mu\text{g ml}^{-1}$ for cisplatin (Pfeifle *et al*, 1985; Urien and Lokiec, 2004) and about $0.02\text{--}0.6\ \mu\text{g ml}^{-1}$ for doxorubicin (Barpe *et al*, 2010; Greene *et al*, 1983). Representative pictures of histological sections from each treatment group are shown in Fig 3.7.

Follicles were assessed morphologically and classified into follicle stage and health. The total number of follicles counted for each treatment group is shown in Table 3.1. The proportion of follicles deemed morphologically unhealthy significantly increased with dosage for both cisplatin and doxorubicin (Fig 3.8.Ai and Bi). To assess whether drug treatment led to follicle loss, total follicle number was calculated from histological analyses and log transformed for normality. Both cisplatin and doxorubicin caused significant follicle loss, but with different patterns of dose response (Fig 3.8.Aii and Bii). Cisplatin induced significant loss of follicles only at

the highest concentration (5 μ g/ml; $p<0.01$), which induced poor health in almost all follicles (Fig 3.8.Ai). In contrast doxorubicin resulted in a significant decrease in follicle number even at a dose which induced poor health in only around 30-35% of follicles (0.05 μ g/ml; $p<0.05$; Fig 3.8.Bi).

As both drug treatments led to increased numbers of unhealthy follicles, they were further categorized to see if a particular follicle class was affected by each drug. Both drugs caused a reduction in primordial follicle number to the point where, at the highest doses there were insufficient present to meaningfully analyse primordial follicle health (mean \pm sem number of primordial follicles in 5 μ g/ml cisplatin group=4 \pm 4, and in 0.2 μ g/ml doxorubicin group=1 \pm 1, vs control=60 \pm 20; Fig 3.9.). However, further analysis was possible for transitional and primary follicles. Cisplatin caused a significant increase in the percentage of morphologically unhealthy transitional follicles only at the highest dose of cisplatin used (5 μ g/ml; $p<0.001$), whilst there were a significant percentage of unhealthy primary follicles at all doses ($p<0.001$; Fig. 3.10.A). In contrast, doxorubicin caused a significant increase in the percentage of both transitional and primary follicles classified as morphologically unhealthy at the second lowest dose ($p<0.05$ for transitional follicles, $p<0.001$ for primary follicles; Fig 3.10.B).

In order to determine which specific follicular cell type was targeted by the two drugs, follicles were further classified as unhealthy due to: (a) the oocyte only; (b) granulosa cells only; or (c) both the oocyte and granulosa cells. In ovaries treated with cisplatin, unhealthy follicles were classified as such primarily due to oocyte

health (Fig 3.11.A), with significant increases in the percentage of follicles with morphologically unhealthy oocytes seen at all doses of cisplatin used ($p<0.05$). Doxorubicin, in contrast, primarily induced follicles classified as unhealthy due to the granulosa cell health (Fig 3.11.B), with significant increases in the percentage of follicles with morphologically unhealthy granulosa cells seen at the three highest doses ($p<0.001$). For both drugs, follicles in which both the oocyte and granulosa cells were unhealthy were rarely seen except at the highest dose (Fig 3.11.C; $p<0.001$ for the two highest dose of both cisplatin and doxorubicin).

During the analysis, it was noted that oocytes appeared larger in ovaries treated with doxorubicin, particularly at the higher doses. To assess this further, oocytes were measured in the ovaries treated with the highest dose of cisplatin and doxorubicin and compared to control (Fig 3.12.). There was a trend towards larger oocytes in doxorubicin treated ovaries; however this was not significant. Oocytes from primary follicles in cisplatin treated ovaries were, though, found to be significantly smaller than control ($p<0.05$). Follicle size was larger in doxorubicin cultures and smaller in cisplatin treated ovaries compared to the control; however this was not significant (Fig 3.12.ii). Nonetheless, it could be that the oocyte diameter is smaller in the primary follicles either because they are less developed or due to their poor health following cisplatin treatment.

	Total number of follicles counted	Total number of follicles classified as healthy	Total number of follicles classified as unhealthy (%)
Control	1572	1513	59 (4)
0.05µg/ml cisplatin	1788	1698	90 (5)
0.1µg/ml cisplatin	1313	968	345 (26)
0.2µg/ml cisplatin	973	824	149 (15)

Table 3.1. Total number of follicles counted in newborn mouse ovaries cultured for 6 days with cisplatin in each treatment group in Experiment 3.1 as well as the total number of these follicles classified as healthy or unhealthy (n=4 ovaries in each treatment group except for 0.2µg/ml cisplatin group where n=2).

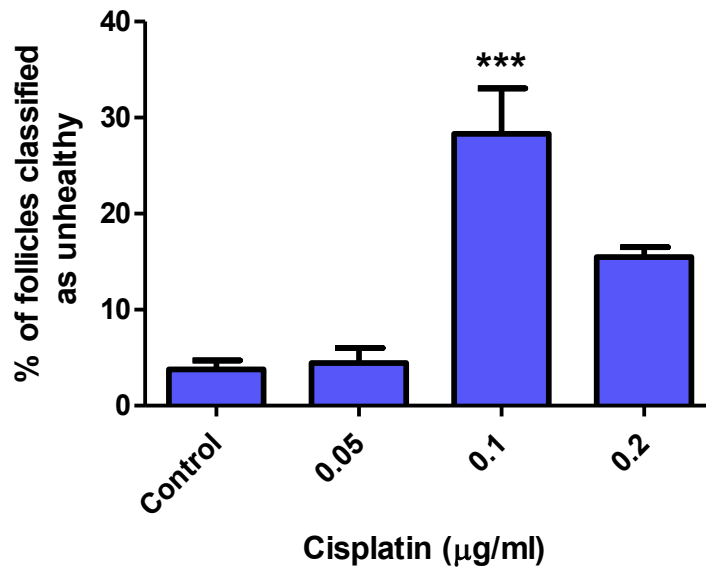


Figure 3.2. The percentage of murine follicles classified as unhealthy following cisplatin exposure. Bars denote mean±sem; n=4 for all groups except 0.2μg/ml where n=2, stars denote significant differences relative to control (***)p<0.001).

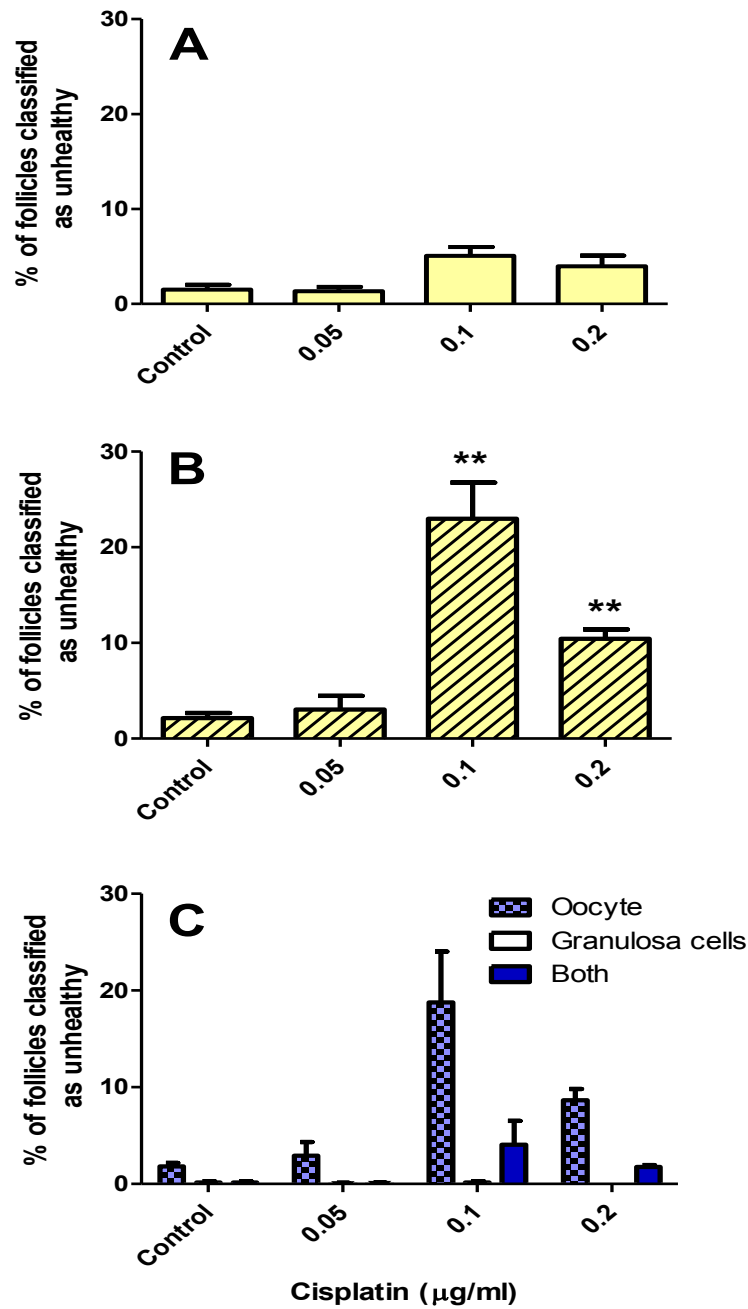


Figure 3.3. The percentage of (A) transitional and (B) primary murine follicles classified as unhealthy following treatment with cisplatin. Primary follicles were further classified into damaged cell type (C): oocyte (blue check), granulosa cells (clear) or both (dark blue). Bars denote mean±sem, n=4 in all groups except 0.2μg/ml where n=2, stars denote significant differences (**p<0.01).

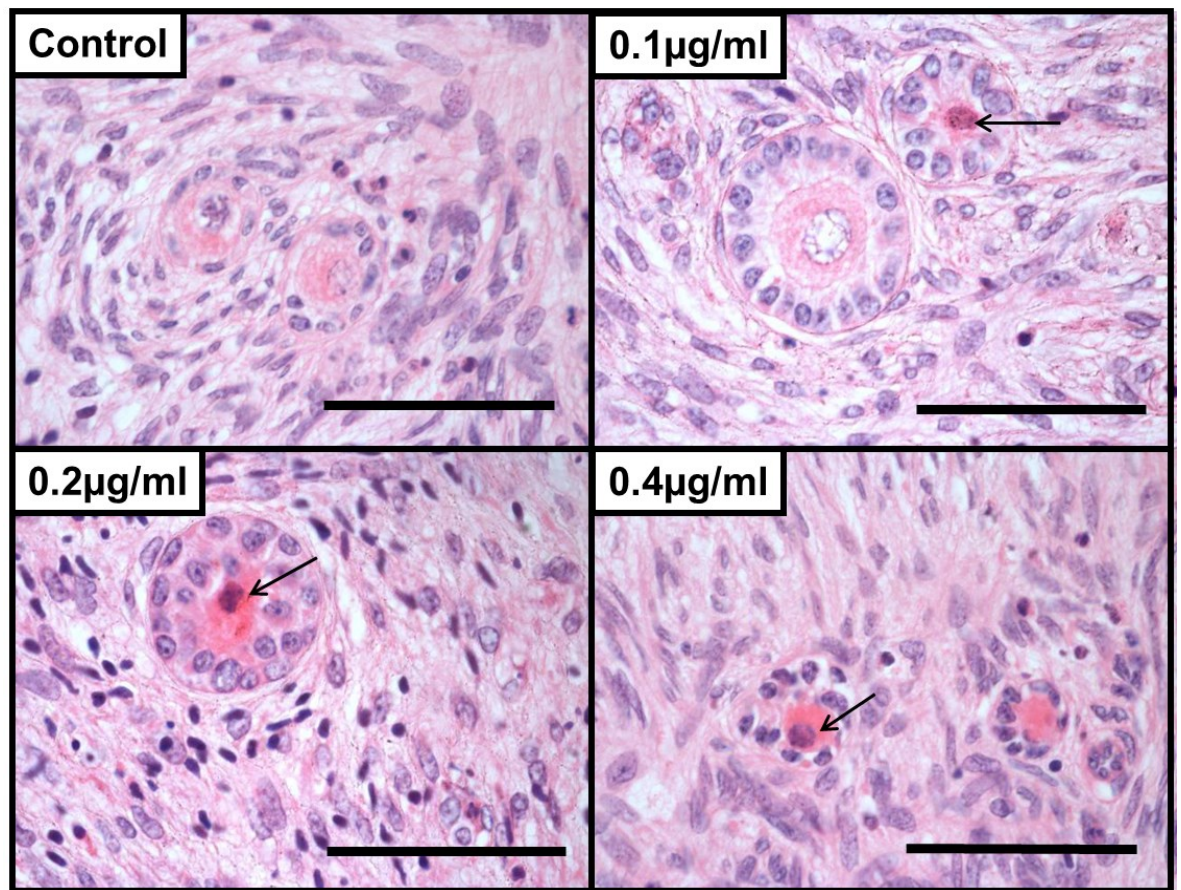


Fig 3.4. Representative histological sections of bovine cortical strips cultured in control, 0.1µg/ml, 0.2µg/ml or 0.4µg/ml cisplatin. Scale bars represent 50µm. Black arrows indicate unhealthy oocytes.

	Total number of follicles counted	Total number of follicles classified as healthy	Total number of follicles classified as unhealthy (%)
Control	1155	865	290 (25)
0.1µg/ml cisplatin	1401	726	675 (48)
0.2µg/ml cisplatin	1179	117	1062 (90)
0.4µg/ml cisplatin	954	207	747 (78)

Table 3.2. Total number of follicles counted in bovine cortical strips cultured for 6 days in the presence of cisplatin as well as the total number of these follicles classified as healthy or unhealthy (n=12 cortical strips in each treatment group).

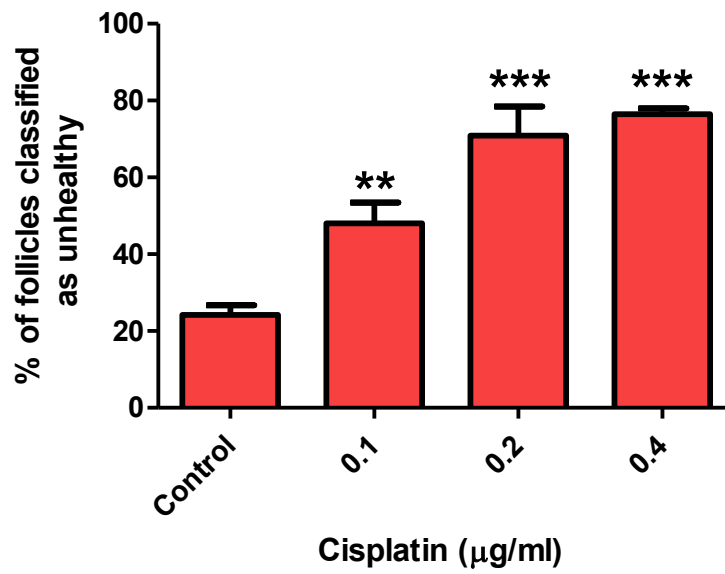


Figure 3.5. Percentage of unhealthy follicles in bovine cortical strips following exposure to cisplatin, n=12 for all groups, bars denote mean±sem, stars denote significant differences relative to control.

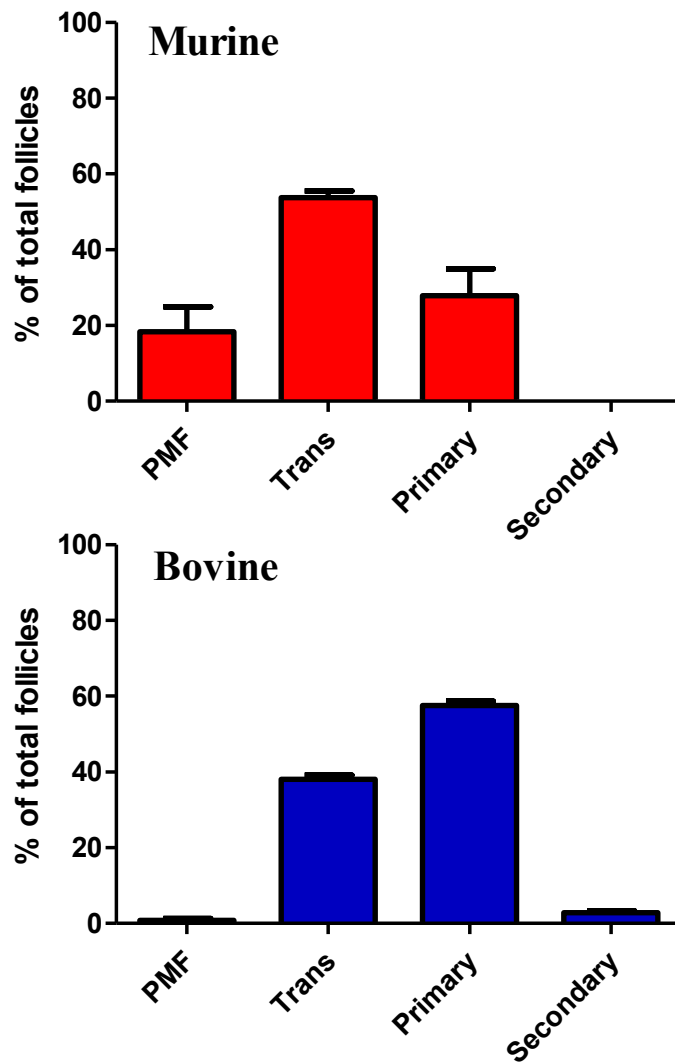
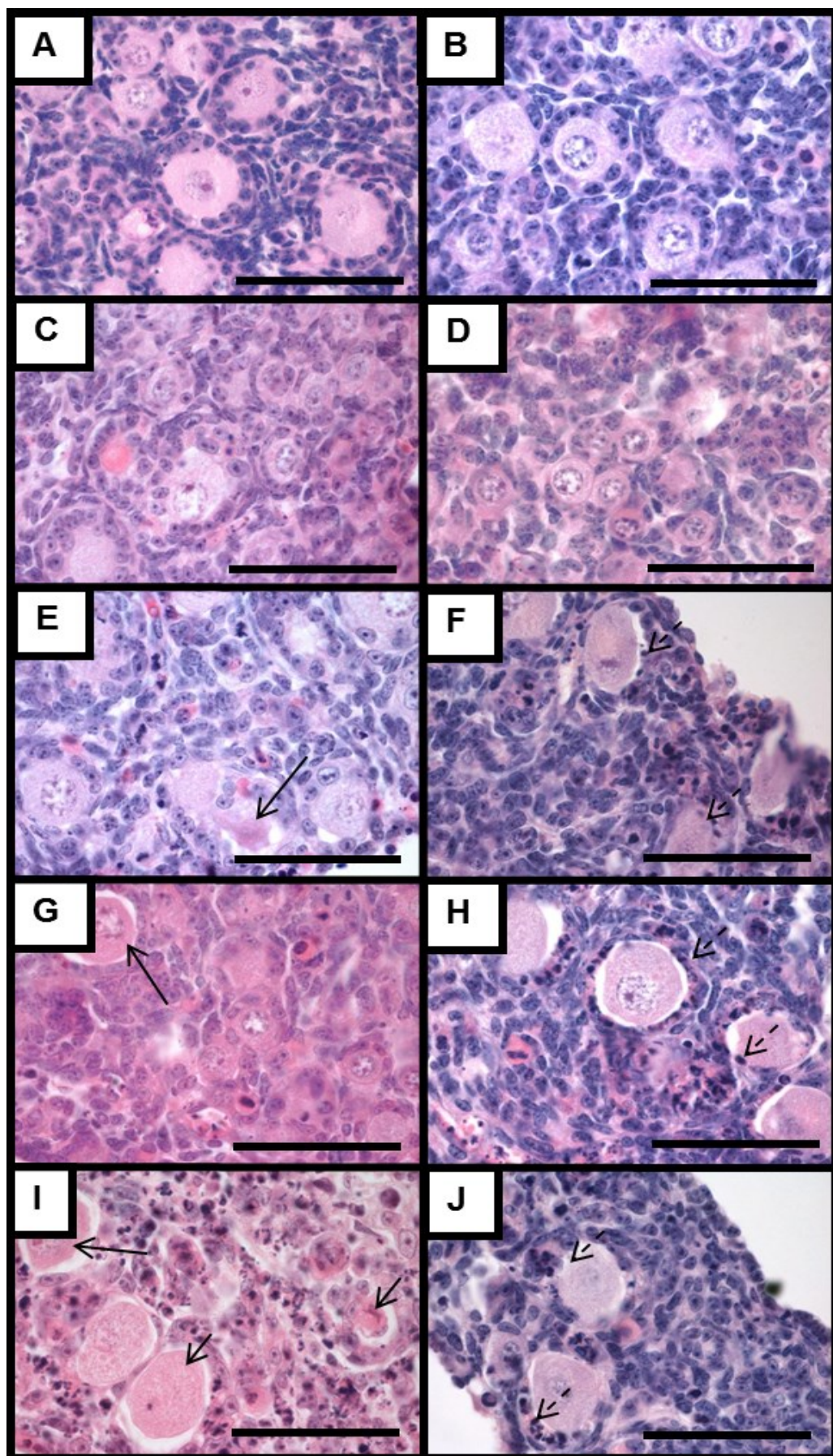


Fig 3.6 Comparison of follicle distribution following six day culture for control treated murine (n=5) and bovine (n=12) tissue. Bars denote mean \pm sem.

Fig 3.7. Representative histological sections of mouse newborn ovaries cultured in increasing concentrations of cisplatin (left column) or doxorubicin (right column). Specifically, **A** and **B**) control, **C**) 0.1µg/ml cisplatin, **D**) 0.01µg/ml doxorubicin, **E**) 0.5µg/ml cisplatin, **F**) 0.05µg/ml doxorubicin, **G**) 1µg/ml cisplatin, **H**) 0.1µg/ml doxorubicin, **I**) 5µg/ml cisplatin and **J**) 0.2µg/ml doxorubicin. Scale bars represent 50µm. Solid black line arrows indicate follicles with unhealthy oocytes. Dashed line arrows indicate follicles with unhealthy granulosa cells.



	Total number of follicles counted	Total number of follicles classified as healthy	Total number of follicles classified as unhealthy (%)
Control	1620	1528	92 (6)
0.1µg/ml cisplatin	1134	900	234 (21)
0.5µg/ml cisplatin	1371	1028	343 (25)
1µg/ml cisplatin	641	385	256 (40)
5µg/ml cisplatin	123	10	113 (92)

	Total number of follicles counted	Total number of follicles classified as healthy	Total number of follicles classified as unhealthy (%)
Control	1574	1522	52 (3)
0.01µg/ml doxorubicin	1667	1532	135 (3)
0.05µg/ml doxorubicin	553	369	184 (33)
0.1µg/ml doxorubicin	286	123	163 (57)
0.2µg/ml doxorubicin	117	19	98 (84)

Table 3.3. Total number of mouse follicles counted in each treatment group in Experiment 3.3 as well as the total number of these follicles classified as healthy or unhealthy (n=5 ovaries in each treatment group).

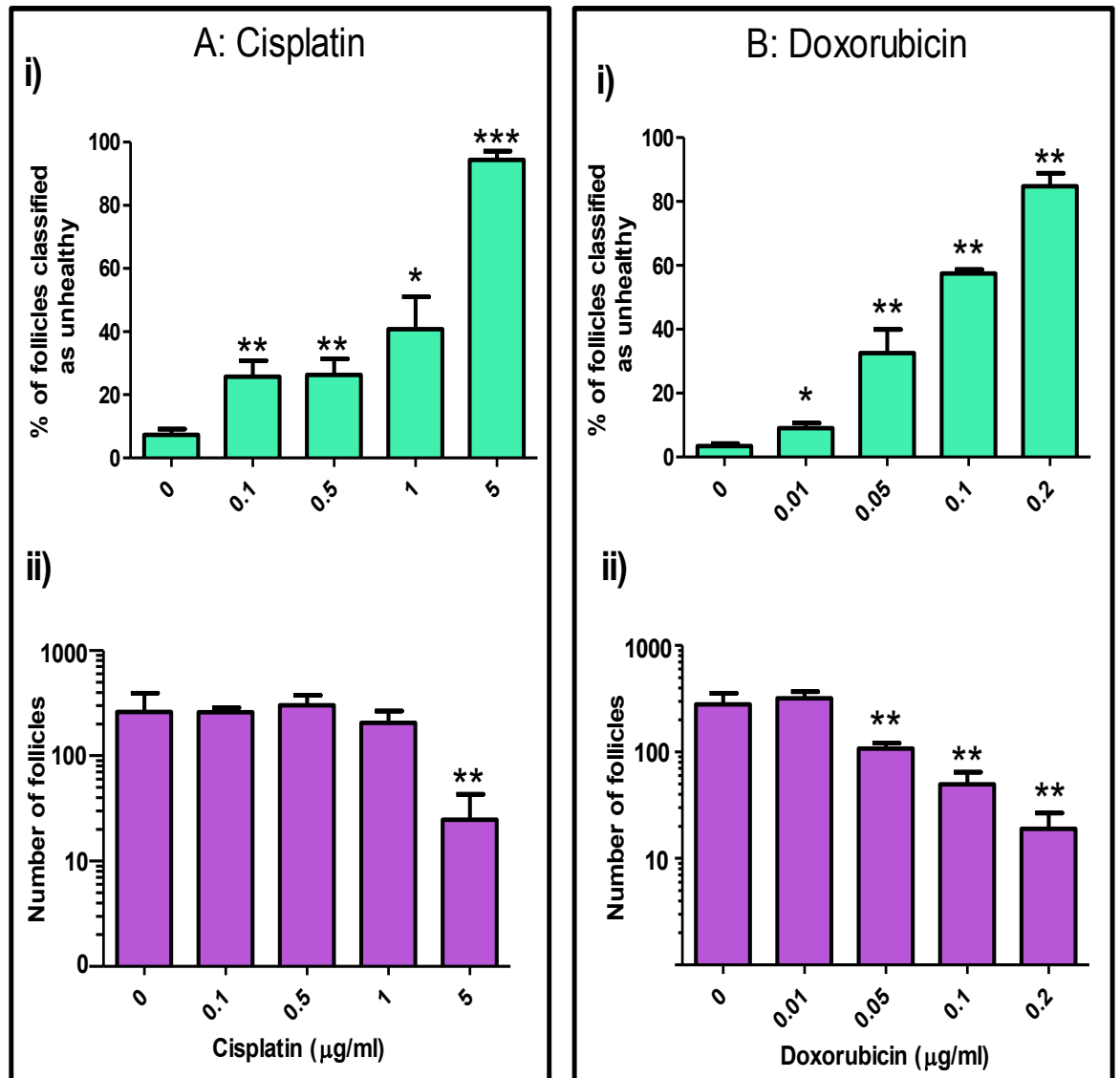


Fig 3.8. (A) Cisplatin and (B) doxorubicin both lead to loss of murine follicle health and a reduction in follicle numbers. (i) Percentage of unhealthy follicles (green) and (ii) total number of follicles (purple) in each ovary. Bars denote mean±sem; n=5 for all groups, stars denote significant differences relative to control (*p<0.05, **p<0.01, *p<0.001).**

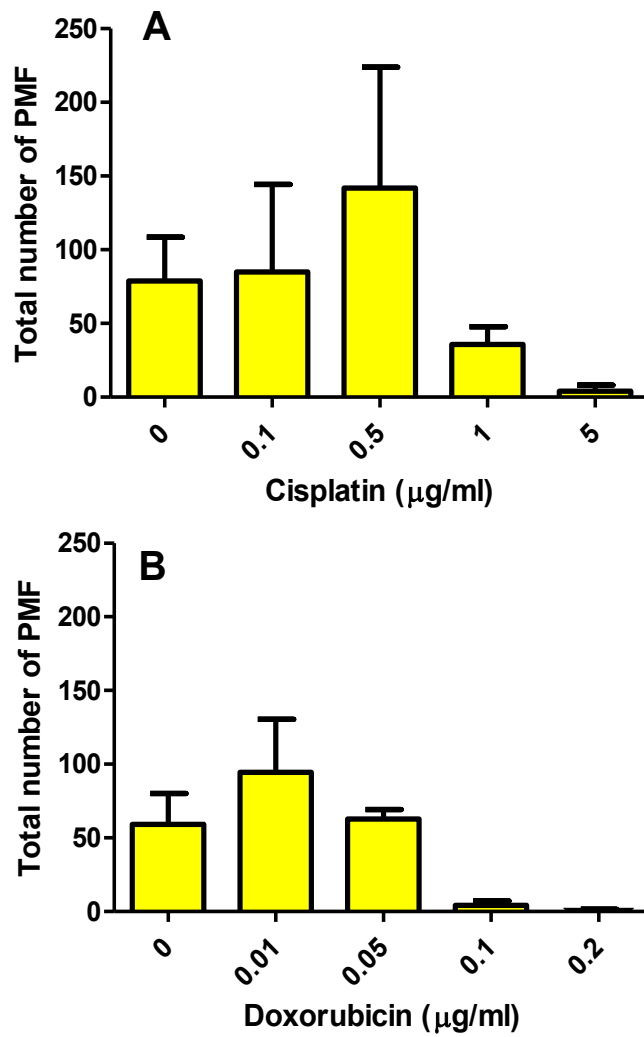


Figure 3.9. Number of primordial follicles (PMF) remaining following treatment of mouse ovaries with **(A)** cisplatin or **(B)** doxorubicin. Bars denote mean \pm sem, n=5.

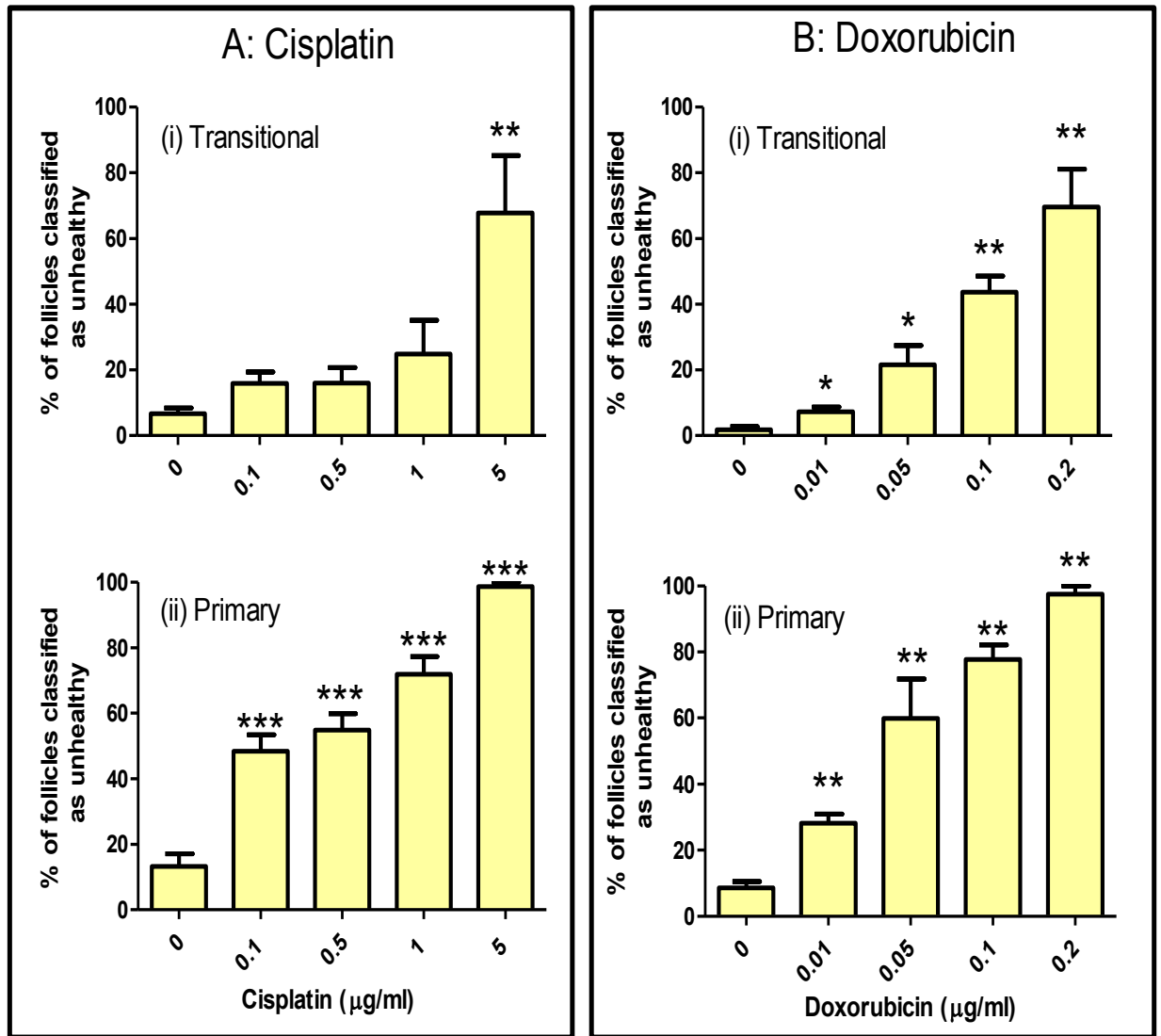
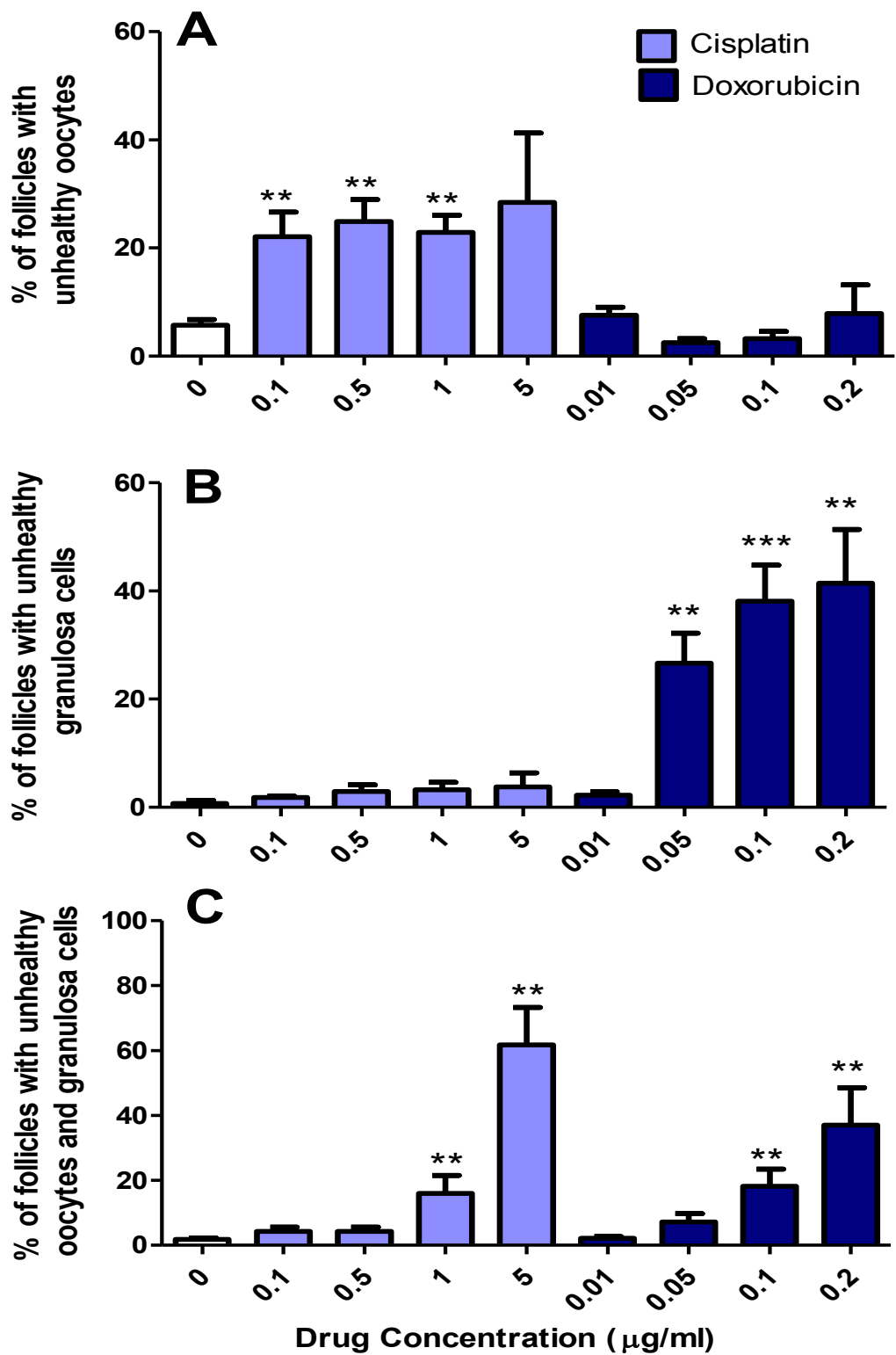


Figure 3.10. Follicles were classified as morphologically unhealthy in cisplatin and doxorubicin treated mouse ovaries according to follicle type. Effect of **(A)** Cisplatin or **(B)** Doxorubicin on the percentage of transitional and primary follicles classified as morphologically unhealthy. Bars denote mean \pm sem; n=5 for all groups, stars denote significant differences relative to control (*p<0.05, **p<0.01, ***p<0.001).

Figure 3.11. Cisplatin and doxorubicin affect different follicular cell types. Mouse ovaries were treated with cisplatin or doxorubicin and all unhealthy growing (transitional and primary) follicles were further categorized as unhealthy due to: **(A)** poor oocyte health; **(B)** poor granulosa cell health; or **(C)** both. Bars denote mean \pm sem; n=5 for all groups except for control where n=10, stars denote significant differences relative to control (**p<0.01, ***p<0.001)



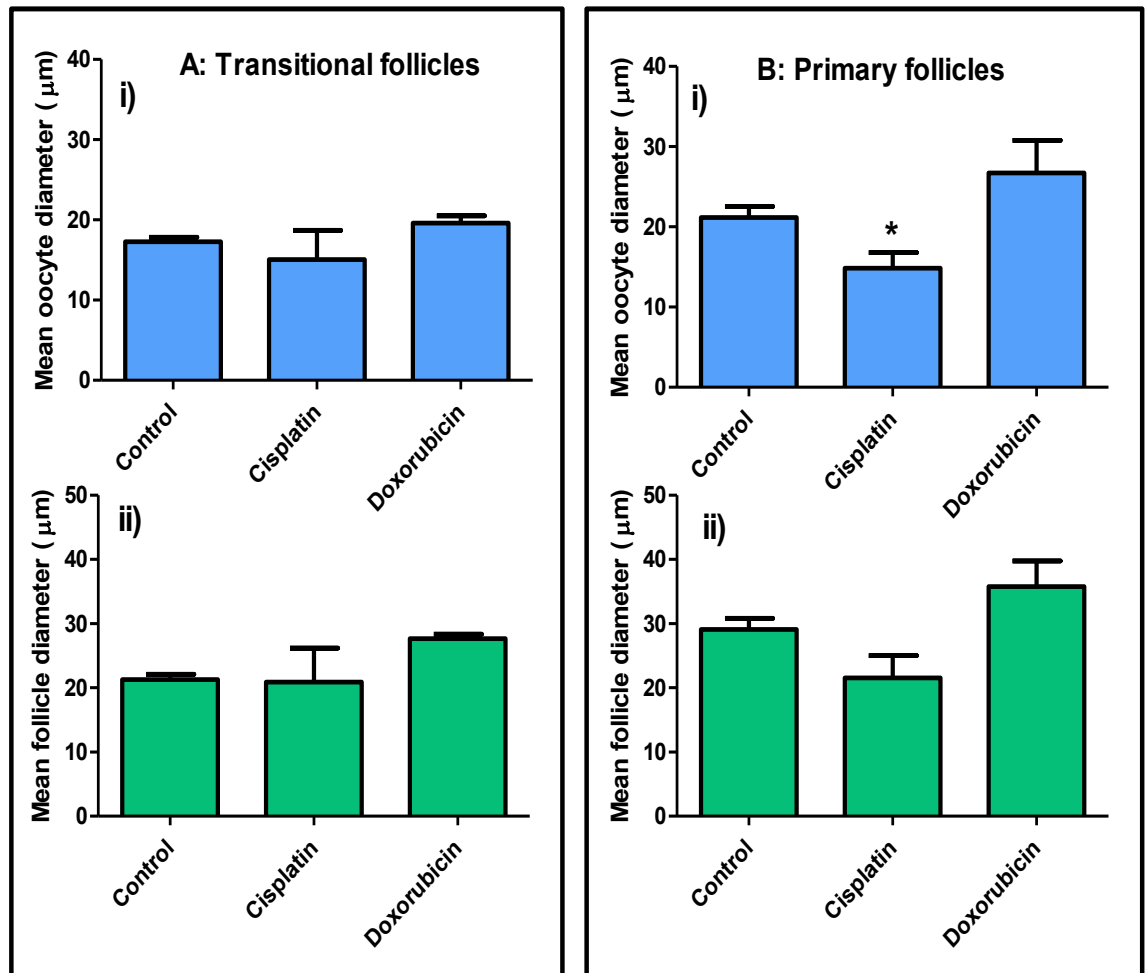


Figure 3.12. (i) Oocyte and (ii) follicle size of **A)** transitional and **B)** primary follicles. Bars denote mean±sem, n=5, stars denote significant differences from control (*p<0.05).

3.4 Discussion

Initial experiments with cisplatin indicate in both the murine and bovine model that cisplatin causes follicle damage, with the oocyte appearing particularly susceptible. When cisplatin and doxorubicin are compared in the same culture model, the two drugs appear to target different cell types.

3.4.1 Comparison of bovine and murine models

When compared to the newborn mouse ovary, the follicle population within bovine cortical strips was different, with the vast majority of follicles activated by the end of the six day culture. Within the newborn mouse ovary culture, a high proportion of primordial follicles activated over the six days but this is not as widespread as in the bovine tissue. This is similar to other studies which have cultured bovine cortical strips (Fortune *et al*, 2000). The reason for such a widespread activation in bovine tissue is unclear but could be due to withdrawal of an inhibitory signal (which remains intact in the mouse whole ovary culture) or due to differences in the nutrient and oxygen exposure. *In vivo*, primordial follicles are maintained in the ovarian cortex which is poorly vascularized and so this increase in nutrient or oxygen exposure could lead to increased follicle activation (Wandji *et al*, 1997).

Whilst the follicle population of the two culture systems was slightly different, there was still a detrimental effect on follicle health in the bovine tissue following cisplatin treatment, with a dose dependent increase in the number of unhealthy follicles. The percentage of follicles classified as unhealthy following drug treatment appears much higher in the bovine cortical strips when compared to similar concentrations of

cisplatin in the preliminary mouse culture, which could indicate that bovine follicles are more vulnerable to cisplatin-induced damage. However, the percentage of follicles which were classified as unhealthy in bovine tissue cultured in control medium is also higher than in the mouse, indicating that the follicles did not survive the culture process as well generally and so may help explain this discrepancy.

In the bovine cultures, the oocyte was also the primary reason that follicles were classified as unhealthy; pyknotic granulosa cells were rarely seen. This indicates that the effect of cisplatin on oocytes noted here is not species specific, and may demonstrate a more general vulnerability of oocytes to cisplatin-induced damage. It is known that mouse oocytes are more sensitive to low doses of radiotherapy than other species (Adriaens *et al*, 2009); it is therefore interesting that the detrimental effect of cisplatin is similar in both species examine here. There is always the concern when using different species (such as the mouse) as a model for humans that results are not transferable due to species related differences; therefore to see similar results in both murine and bovine tissue is quite reassuring in terms of how applicable our model is. Ideally the next step would be to culture human tissue with cisplatin or doxorubicin to check if similar results are seen; this is currently being done in the lab.

3.4.2 Comparison of cisplatin and doxorubicin

Both cisplatin and doxorubicin have been linked to premature ovarian failure due to follicle loss; data presented here is consistent with this as there was a decrease in follicle number and an increase in unhealthy follicles following both drug treatments

when compared in the same murine ovary model. Both drugs were used in doses within serum range for patients which are around 0.5-1 $\mu\text{g ml}^{-1}$ for cisplatin (Pfeifle *et al*, 1985; Urien and Lokiec, 2004) and about 0.02-0.6 $\mu\text{g ml}^{-1}$ for doxorubicin (Barpe *et al*, 2010; Greene *et al*, 1983).

The stage of follicle loss affected following cisplatin treatment is predominantly the growing primary follicles in contrast to doxorubicin which equally affects both transitional and primary. Morphologically, it was difficult to assess the health of primordial follicles. By the highest doses of drugs, primordial follicle numbers were too low for a meaningful statistical assessment. Assessing the ovaries at an earlier point in culture may be a way around this, as the predominant follicle class in the first 3 days of culture is primordial (Federica Lopes, unpublished data, Fig 3.1). Throughout the six days, more primordial follicles are activated and so there is a shift in the class distribution. Primordial follicle numbers could be depleted here due to direct primordial follicle damage by the chemotherapeutic agents or due to an indirect effect; damage to more mature, growing follicles would lead to increased recruitment of primordial follicles out of the resting pool and hence to premature depletion of that resting follicle reserve (Meirow *et al*, 2010; Morgan *et al*, 2012).

3.4.2.1 The effect of cisplatin

Cisplatin causes cell death through DNA crosslinking and adduct formation. When the DNA damage is overwhelming, this activates cell signalling pathways culminating in apoptosis. Following cisplatin exposure, the primary reason for classifying a follicle as unhealthy was the oocyte, suggesting that cisplatin directly

targets the germ cell. This corroborates the preliminary data in both the murine and bovine models, which also observed oocyte specific damage. Given the importance of protecting the integrity of the germ line from DNA damage, it is perhaps logical that the germ cell may be particularly vulnerable to agents that cause overwhelming DNA damage. The sensitivity of the oocyte to cisplatin in particular could be explained by the diffuse chromatin in oocytes (Luciano *et al*, 2012). A study which exposed Hela cells to cisplatin and examined alterations in gene expression found that cisplatin inhibited expression of genes which were associated with more accessible chromatin (Jamieson and Lippard, 1999). The preferential site of ovarian toxicity being the oocyte may explain why follicle health was not reduced in follicles on initiation of growth (i.e. transitional follicles), but manifested at the slightly later primary stage. The fact that oocytes were significantly smaller in primary follicles at the highest dose is likely a reflection of their poor health; one of the major criteria for assessing oocyte health is shrunken cytoplasm. As discussed above, there is a larger percentage of primary follicles present in bovine cortical strips. If there is an increased vulnerability in follicles which have been activated to grow following cisplatin exposure, this may be another explanation of why the cortical strip cultures had such a high proportion of follicle death.

3.4.2.2 The effect of doxorubicin

Doxorubicin causes cell death through inhibition of topoisomerase enzymes and causing problems in DNA replication and transcription. Results here show that doxorubicin may directly target the granulosa cells, as the primary reason for assessing follicles as unhealthy was due to pyknotic granulosa cells. In contrast to

oocytes, granulosa cells are mitotically active, which may explain their vulnerability to these types of chemotherapy agents. Mature (MII) oocytes which are exposed to doxorubicin *in vitro* undergo apoptosis (Jurisicova *et al*, 2006; Perez *et al*, 1997) and oocytes collected from antral follicles and cultured are also highly susceptible to such damage (Bar-Joseph *et al*, 2010). As the oocytes examined here are not mature but contained within immature follicles, they may be less sensitive to doxorubicin. However, the model used here may be of more relevance to patients, as the oocytes contained in immature follicles are required to maintain long term fertility. A study in human primordial follicles treated *in vitro* to doxorubicin observed damage in both the granulosa cells and oocyte; however this study used much higher concentrations of doxorubicin than found in patient serum (Soleimani *et al*, 2011). The slightly larger oocyte size seen in doxorubicin treated ovaries, although not significant, could be a real effect, perhaps due to a dysregulation of oocyte growth by the granulosa cells. Increasing the n number would be useful here, to see if the result becomes significant. Due to the essential interaction between the oocyte and granulosa cells for each others growth and development, any problems in the granulosa cells could have a downstream effect on oocyte growth and health.

3.4.3 Stromal effects

Some research has suggested that chemotherapy may damage the stroma, and that this damage may have a detrimental downstream effect on follicle health (Marcello *et al*, 1990; Meirow *et al*, 2007). There was pyknosis of stromal cells evident in both the mouse and bovine models but this was inconsistent between treatment groups. Molecular analysis of cell death markers may be a more meaningful and practical

method of assessing any detrimental effects of chemotherapy on the ovarian stroma and will be discussed further in Chapter 4.

3.4.4 Conclusion

The data presented here indicate that cisplatin and doxorubicin cause ovarian follicle loss but target different cells. The molecular mechanisms through which these drugs cause differential effects are unclear and will be explored further in Chapters 4 and 5.

CHAPTER 4

MOLECULAR MECHANISMS BY WHICH CISPLATIN AND DOXORUBICIN INDUCE CELL DEATH

4.1 Introduction

There are multiple potential pathways through which a cell can die, with the considerable cross-talk between these making dissecting specific mechanisms difficult. The ovary is the site of a large amount of cell death physiologically, with the vast majority of follicles dying through atresia. The exact molecular pathways involved in follicle atresia are not well understood, with some studies implicating apoptosis and others indicating autophagy. Chemotherapy could induce ovarian follicle death through similar pathways as follicle atresia, or it could act through alternate mechanisms.

4.1.1 Apoptosis

Apoptosis is perhaps the most well defined mechanism of cell death. The term programmed cell death was often previously used synonymously for apoptosis; however there is now more recent evidence that other cell death pathways such as necrosis and autophagy are also forms of programmed cell death. Apoptosis plays a significant role during development and is responsible for the removal of cells which are potentially harmful, senescent or no longer needed. Morphologically, it is characterised by membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation. Apoptosis can be induced through pro-death signalling, a lack of growth factor signalling or anti-apoptotic signalling as well as stress inducers such as radiation. There is some evidence that apoptosis is involved in follicle loss following chemotherapy treatment (Perez *et al*, 1997) and a few specific signalling cascades have been investigated. One such is the ceramide pathway, which when suppressed can prevent oocyte apoptosis (Morita *et al*, 2000).

4.1.1.2. Markers of apoptosis

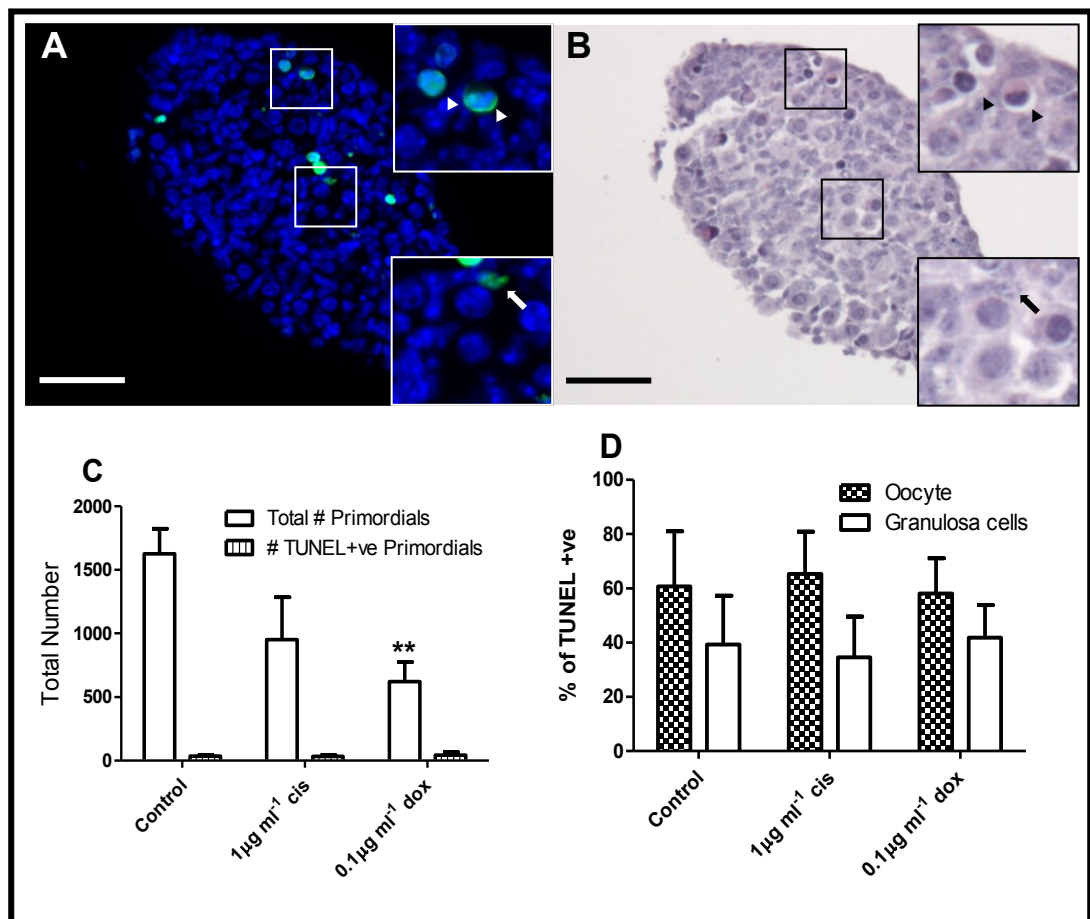
There are several markers which are often used to detect evidence of apoptosis. These include TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labelling) assays, which detects DNA fragmentation, cleavage of the caspase protein family (traditionally Caspase 3) and cleavage of PARP-1 (Poly [ADP-ribose] polymerase 1).

The PARP family of enzymes cause poly(ADP-ribosyl)ation, which is important for cell survival and the cell stress response. It is the process of adding long chains of ADP-ribose onto proteins as a form of post-translational modification. Proteins which are modified in this way include topoisomerases, histones, DNA polymerases and ligases. There are several members of the PARP family but by far the best characterised is PARP-1, which is activated predominantly in the presence of single and double strand DNA breaks (Virag *et al*, 2013). PARP-1 has been demonstrated to be essential for DNA repair during genotoxic stress in both PARP-/- mice (de Murcia *et al*, 1997) and cell lines (Trucco *et al*, 1998). When DNA damage is minor, PARP activates enzymatic machinery such as DNA polymerase and ligase, allowing repair of these breaks (Soldani and Scovassi, 2002). Where DNA damage is extensive, hyperactivation of PARP can lead to a large increase in poly(ADP-ribose) levels, an energy expensive process as it occurs at the expense of the cells NAD^+ (Nicotinamide adenine dinucleotide) supply (Burkle and Virag, 2013). The decrease in NAD^+ causes an imbalance in the NAD/NADH ratio which affects the activation of enzymes involved in respiration, as well as altering the redox state of the cell. The

cell will then try to restore the NAD pool by recycling nicotinamide with ATP (Adenosine triphosphate), meaning that excessive activation of PARP-1 depletes the pool of ATP and NAD^+ (Boulares *et al*, 1999), affecting all energy-dependent processes in the cell. PARP-1 is cleaved during apoptosis, predominantly by the caspase family of proteins, in order to conserve the energy supplies of the cell.

In work by others in this laboratory (Lopes and Spears), TUNEL staining was used to determine if there was an increase in DNA fragmentation in primordial follicles following treatment of ovaries with either cisplatin or doxorubicin. There was no significant increase in the number of cells labelled TUNEL positive (Fig 4.1), suggesting that apoptosis may not be involved in cisplatin or doxorubicin induced primordial follicle loss (Morgan *et al*, 2013, work done by Federica Lopes).

Fig 4.1 TUNEL-analysis to determine apoptosis in primordial follicles. **(A):** ovary section labelled with TUNEL reaction (green) and counterstained with DAPI (blue). Inset top- and bottom-right magnification images are of the respective framed areas, illustrating examples of TUNEL positive oocytes (white arrowheads) and TUNEL positive granulosa cell (white arrow) within follicles identified as at the primordial stage in (B). **(B):** section in (A) subsequently stained with haematoxylin and eosin. Inset sections in (A) correspond here to degenerated oocytes (black arrowheads) and degenerated surrounding granulosa cells (black arrow) within primordial follicles. Scale bar = 50 μ m. **(C)** Total number of primordial follicles, and number of primordial follicles containing TUNEL-positive cells, in ovaries treated with cisplatin or doxorubicin. **(D):** TUNEL-positive primordial follicles further categorised into percentage in which the oocyte or the granulosa cells stained positive. Bars denote mean \pm sem; n=4-5 ovaries per treatment group; stars denote significant differences relative to control (**P<0.01).



4.1.2 Autophagy

Autophagy is a form of programmed cell death which is highly conserved and tightly regulated. There is a basal level of autophagy present in all eukaryotic cells which helps maintain homeostasis, as it involves the degradation of cytoplasmic components leading to the production of metabolites for the cell. Autophagy is upregulated following amino acid starvation and other cellular stresses. If autophagy is inhibited, there is evidence that this can lead to alternative cell death pathways such as apoptosis. Dysregulation of autophagy has been linked to various diseases such as cancer (Kondo *et al*, 2005) and neurodegeneration. At the molecular level, it involves organelles and components of the cytoplasm being engulfed in autophagosomes (double membraned vacuoles), which then fuse with lysosomes and are degraded (Choi *et al*, 2010).

There are over 30 genes which have been identified as playing an important role in autophagy; some of which are essential for it to occur. These include *Beclin1*, *Ulk1* (unc-51 like autophagy activating kinase 1), *Atg7* (Autophagy-related protein 7), and *Atg13* (Autophagy-related protein 13). *Beclin1* promotes the formation of the autophagosome, allowing for cytoplasmic components to be sequestered and degraded. It also interacts with the canonical apoptosis pathway, by interacting with the anti-apoptotic Bcl-2 family of proteins (Liang *et al*, 1998). *Ulk1* is responsible for phosphorylating *Beclin1*, allowing for formation of the autophagosome and is essential for the induction of autophagy in mammals (Russell *et al*, 2013). *Atg13* is also essential for autophagy induction, at least in part due to its ability to enhance *Ulk1* activity (Alers *et al*, 2011). *Atg7* is required for expansion of the

autophagosome (Mehrpour *et al*, 2010) and in the ovary, a null mutant mouse for *Atg7* lacks any discernable germ cells by postnatal day 1 (Gawriluk *et al*, 2011), indicating its importance in germ cell survival (Summarised in Fig 4.2.).

Autophagy is essential in the *Drosophila melanogaster* ovary for the process of oogenesis to successfully occur (Barth *et al*, 2011). In the mouse ovary, autophagy appears to be an important cell survival mechanism for germ cells, as mice deficient in *Becn1* or *Atg7* have a significant reduction in the number of primordial follicles present perinatally (Gawriluk *et al*, 2011). This suggests that autophagy may be important in regulating germ cell nest breakdown and/or primordial follicle formation. In a toxicology test investigating the ovotoxicity of cigarette smoke, autophagy was found to be the key mechanism of follicle loss following such exposure (Gannon *et al*, 2012).

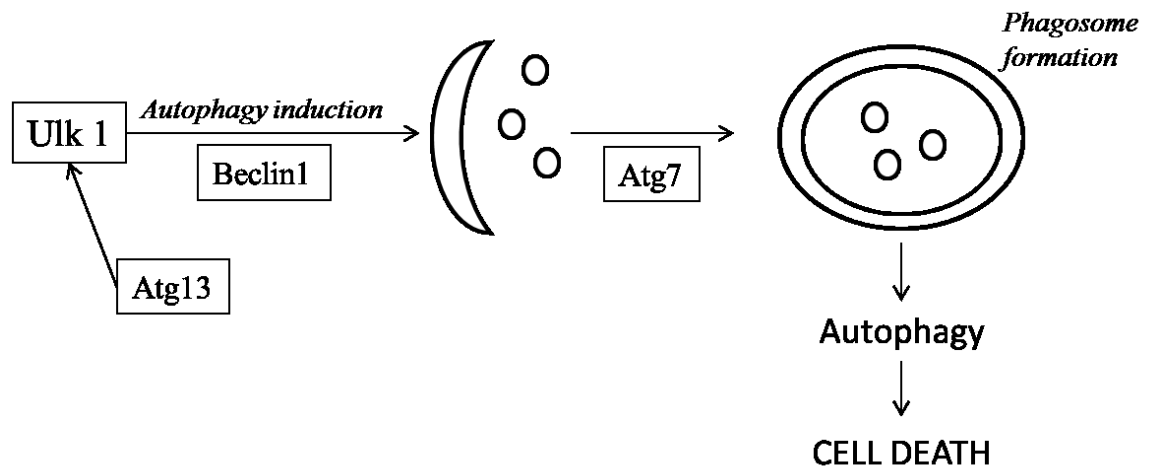


Fig 4.2. Brief summary of autophagy. *Ulk1* is the main gene responsible for the induction of autophagy, its activity being enhanced by *Atg13*. Through *Bcln1* and *Atg7* the phagosome is formed, allowing for the degradation of cytoplasmic components.

4.1.3 Necrosis

Another process of programmed cell death is necrosis, which involves characteristic cell changes including increased membrane permeability and nuclear degeneration (de Bruin *et al*, 2002). Necrosis was not considered to be a form of programmed cell death until recently; previously, it was considered to be more of an ‘accidental’ or passive response to cell injury. The mechanisms of necrosis and apoptosis have massive overlap, with many key players involved in both processes. Necrosis can be induced by cytokines such as Tumour necrosis factor (TNF) and FasL, ischemia, oxidative stress or DNA damage (Proskuryakov *et al*, 2003). The reason that a cell enters into necrotic cell death rather than apoptosis is not clear but is likely related

mainly to the energy status of the cell (Eguchi *et al*, 1997) as well as the cell type and its environment. Apoptosis is an ATP-dependant process, whilst an energy deficiency such as a large reduction in cell ATP can lead to the alternative necrotic pathways. The importance of necrosis in the ovary is not as well defined as apoptosis but has been linked to the process of ovulation in sheep (Murdoch *et al*, 1999).

Due to the massive overlap between necrotic and apoptotic intracellular pathways, it is difficult to solely identify the activation of necrosis. Most experimental techniques rely heavily on the morphological differences between the two, as necrosis causes cells to swell and the membrane to become more permeable (Proskuryakov *et al*, 2003). This is in contrast to apoptosis which causes cells to shrink and nuclei to condense. Work by others in the laboratory (Zukowska, Lopes and Spears, unpublished data) using propidium iodide absorption (to demonstrate increased membrane permeability) as a marker for necrosis found no significant increase following drug treatment, suggesting that this is not a key mechanism of cisplatin or doxorubicin induced follicle death.

4.1.4 Aims

Given that the classes of chemotherapy drugs are all linked to an array of different mechanisms, it is likely that different drugs could induce a variety of specific cell death pathways, meaning that the effect on the ovary could be very drug specific. The aim of the work in this chapter was to compare the molecular actions of cisplatin and doxorubicin and to determine if different pathways are activated. As preliminary

experiments indicated that necrosis may not be involved, work concentrated on autophagy and apoptosis.

4.2 Materials and methods

4.2.1 Animals

C57BL6 newborn female mice were used for all experiments.

4.2.2 Culture of newborn ovaries.

Newborn mouse ovaries were cultured as described in Chapter 2. The culture time was shortened to 2 days, so that ovaries were cultured in control medium for 24 hours, exposed to either cisplatin or doxorubicin for a further 24 hours and then removed from culture. Ovaries were then washed in cold PBS (Phosphate buffered saline, Invitrogen # 10010-015, pH7.4) and snap frozen in eppendorfs on dry ice. For Westerns assessing the time course of cleaved PARP expression, ovaries were removed from culture after 4, 8, 12 or 24 hours of cisplatin exposure (following the initial 24 hours of culture). Each time point had a control and a treated group.

For qPCR analysis, 3 ovaries were pooled in each treatment group to create 1 sample. This is because preliminary experiments indicated that fewer ovaries provided too small a yield of RNA. Three samples were then analysed which were taken from 3 independent cultures. For Western blot analysis, 2 ovaries were pooled in each treatment group to create 1 sample and to ensure sufficient protein yield. For the time course experiments, 2 samples from different cultures were analysed. For the 24 hour time point Westerns, 3 samples from different cultures were analysed.

4.2.3 RNA extraction

RNA was extracted from ovaries using an RNeasy mini kit (Qiagen UK, #74104) and according to manufacturer instructions (Summarised in Fig 4.3). On column DNA digestion was included to remove any genomic DNA contamination. From one eppendorf which contained 3 ovaries of the same treatment group pooled, 30µl of RNA containing water was eluted.

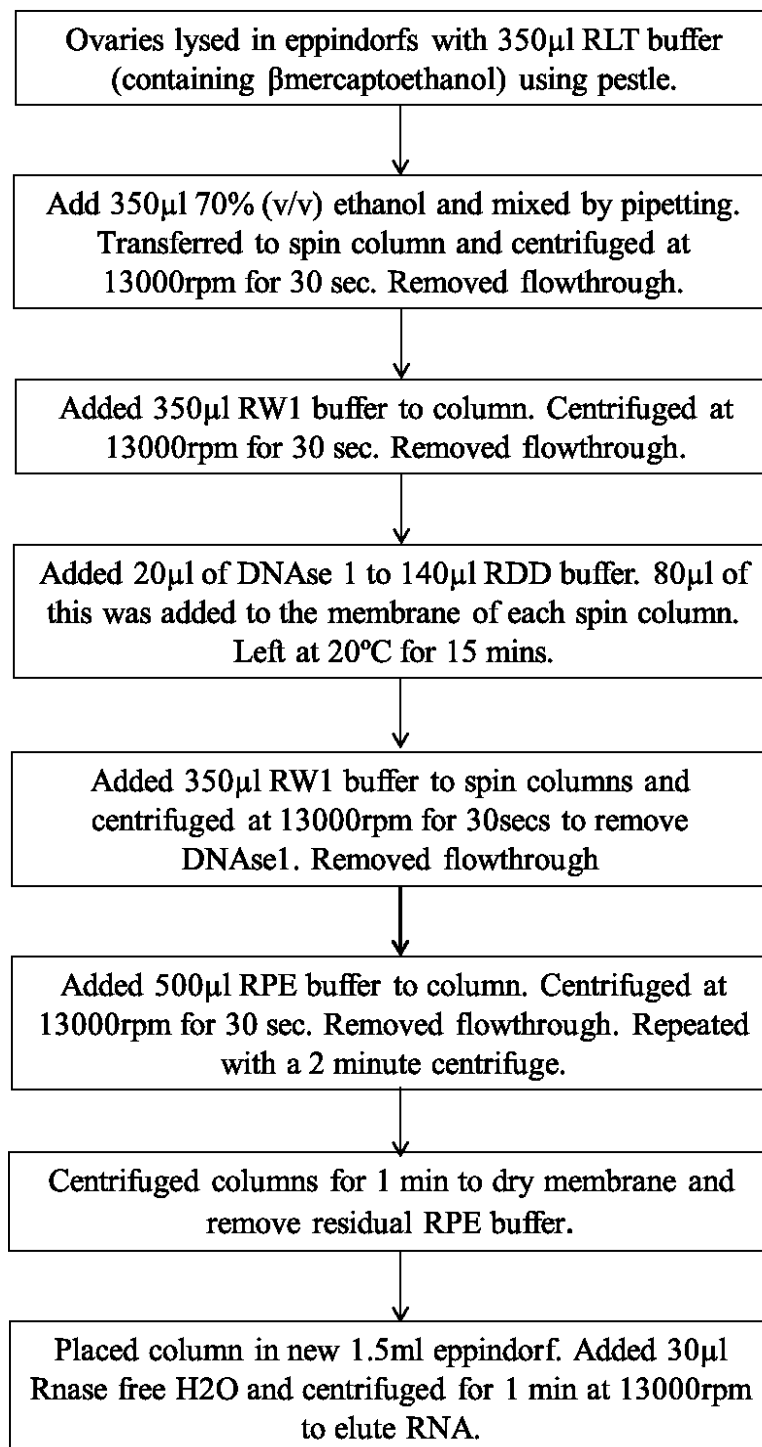


Fig. 4.3. Summary of protocol for RNA extraction using RNeasy mini kit.

The concentration of RNA eluted was assessed using a Nanodrop 2000 (Thermoscientific, UK). The average concentration of RNA expected was

approximately 20-30µg/µl. If the concentration of RNA was too low, the sample was discarded. The quality of eluted RNA was assessed by running 1µl of each sample on a 1% (w/v) agarose gel.

4.2.4. RNA gel

A 1% (w/v) agarose gel was made containing SYBRsafe staining dye (1µl in 50mls, Invitrogen, #S33102). The gel was prepared by dissolving 0.5g agarose (SeaKem® LE agarose, #50002, Stratech Scientific UK) in 50mls 1% TBE buffer. Solution was microwaved and topped up with dH₂O until completely dissolved. Samples were added to a loading dye (6x, #R0611, ThermoFisher), separated using electrophoresis and examined using a UV transilluminator at 254nm. Two clear bands representing 18s and 28s ribosomal RNA were considered good, any smeared samples were discarded.

4.2.5. cDNA synthesis

cDNA was synthesised using the Quantitect Reverse Transcription kit (Qiagen, #205311) and according to manufacturers instructions. RNA was incubated with gDNA Wipeout Buffer to remove any genomic DNA contamination. The RNA was incubated in a mixture of primers, reverse transcriptase enzyme, Mg²⁺ and dNTPs for 15 mins at 42°C. Alongside, a sample of RNA was incubated with the same mix without reverse transcriptase enzyme present. This was to provide an additional control for qPCR analysis (called –RT control). All samples were then heated to 95°C for 3 minutes to inactivate the reverse transcriptase enzyme. Samples were then stored at -80°C until needed (summarised in Fig 4.4).

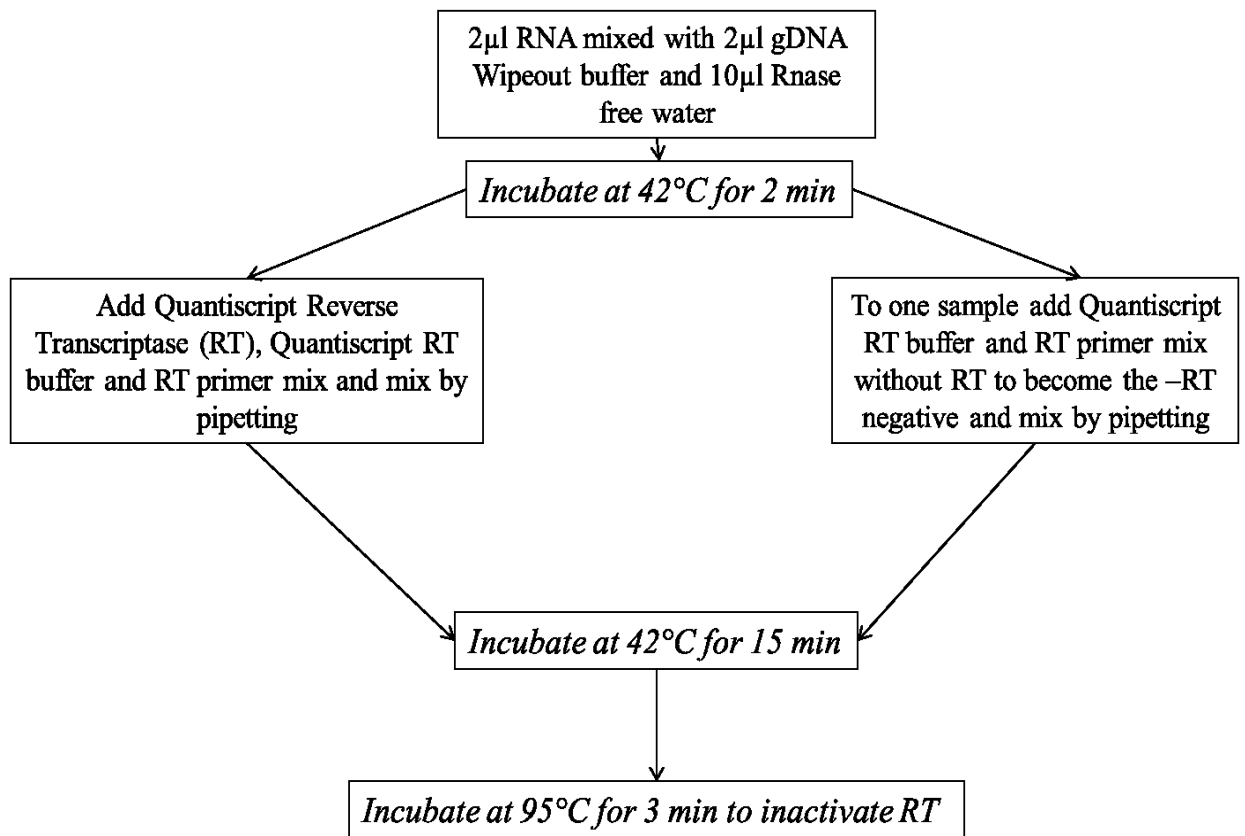


Fig 4.4. Flow diagram of cDNA synthesis protocol using the Quantiscript Reverse Transcription Kit.

4.2.6. Primer design

For every cDNA which was analysed by real time PCR, primers had to be designed. cDNA sequences were obtained from the NCBI Nucleotide Database (<http://www.ncbi.nlm.nih.gov/nuccore/>), including alternatively spliced isoforms. The gene region which was chosen to design primers was common to all alternatively spliced isoforms and when possible, spanned exon exon junctions, to help eliminate the amplification of contaminating genomic DNA during PCR.

Once the gene region was chosen, this was imported into the online primer design software *Primer3* (<http://frodo.wi.mit.edu/>). Primers were selected on the following criteria:

1. Were 18-25 base pairs long (ideally 20)
2. Amplified a region of up to 250 base pairs
3. Had low scores on primer-primer self and inter- complementarity tests
4. Had a GC% ratio of around 50%
5. An annealing temperature range of 55-60°

Each primer sequence was then checked in the NCBI database BLAST algorithm to ensure that the primers were specific to the gene of interest.

Gene of interest	Forward primer	Reverse primer
<i>Becn1</i>	5'AGCCTCTGAAACTGGACACG	5'TGGCGAGTTTCAATAAATGG
<i>Ulk1</i>	5'TGGGCAAGTTCGAGTTCTCT	5'TTGATTTCCTTCCCAGCAG
<i>Atg7</i>	5'CCTGCACAACACCAACACAC	5'CGAAGGTCAGGAGCAGAAAC
<i>Atg 13</i>	5'TTTTGCCCTCAAGACTGTCC	5'GACAGGGCCTTCTTTGCTTC

Table 4.1. Primer sequences designed for qPCR analysis

4.2.7. Quantitative PCR

The relative amount of cDNA in an ovary sample was quantified using the QuantiTect SYBR Green Real-Time PCR Kit (Qiagen, #204143) and according to manufacturer's instructions.

<i>Quantitect SYBR Green Real Time PCR</i>	<i>Quantity/Reaction</i>
<i>Kit Component</i>	<i>(μl)</i>
SYBR Green	10
RNase free H ₂ O	7
cDNA	1
Reverse Primer	1
Forward Primer	1

Table 4.2. Reaction mix details for Real Time qPCR analysis

Reaction mixes (Table 4.2) were made up in sterile PCR tubes and mixed thoroughly by pipetting (#TC50851, lids #TC50803, BioRad). The relative amounts of any genes quantified were normalised against the housekeeping gene *Gapdh* (Glyceraldehyde 3-phosphate dehydrogenase), using the sense 5'GGGTGTGAACCACGAGAAAT and the antisense 5'CCTTCCACAATGCCAAAGTT primers (Primers designed by P Filis). *Gapdh* was selected as a housekeeping control as it is a well-established one and preliminary experiments indicated that it had a constant expression between samples tested. In each experimental run, a sample of cDNA from control, cisplatin and doxorubicin treated ovaries were run in triplicate for the gene being examined and *Gapdh*. Additionally a water and a –RT control was included for each gene. Alongside this, a standard curve for each gene would be run using 5-fold dilutions of highly concentrated cDNA from adult mouse ovaries.

4.2.8. qPCR analysis

The values for each triplicate analysis were averaged and normalised to the housekeeping gene values, to make one value per experimental run. Each gene was analysed in 3 separate runs, with a different ovary sample used in each i.e. an n of 3 for each gene analysed. ANOVA was used to determine if groups were significantly different followed by Bonferroni post hoc tests.

4.2.9. Protein extraction.

20µl of protein lysis buffer (50mM HEPES buffer, 10% [v/v] Triton X, 50mM NaCl, Protease inhibitor cocktail [#11697498001, half a tablet in 10mls] and protease

inhibitors [I, #P0044, 1% (v/v) and II, #P5726, 1% (v/v)], H₂O; all purchased from Sigma Aldrich Ltd except for Protease inhibitor cocktail which was purchased from Roche Diagnostic Ltd, UK) was added to snap frozen ovaries. Ovaries were then homogenised using a sterile pestle and centrifuged then at 4°C for 20 minutes at 13000rpm. 10µl of supernatant was removed and frozen at -20°C until use. Protein extracts were denatured by boiling them for 5 minutes at 95°C in 1:1 Laemmli buffer (Sigma-Aldrich, #127K6052) and loaded into a 7% (v/v) polyacrylamide gel.

4.2.10. Western blotting

7% (v/v) polyacrylamide gels (Resolving gel: 7% (v/v) Acrylamide/bis acrylamide (Sigma Aldrich, #A3699), 0.1% SDS [Sodium dodecyl sulphate], 375mM Tris HCl, 0.15% Ammonium persulfate and 0.0004% TEMED [N,N,N',N'-Tetramethylethylenediamine], Stacking gel: 4% Acrylamide/ bis acrylamide, 0.1% SDS, 125mM Tris HCl, 0.15% Ammonium persulfate and 0.004% TEMED, pH 6.8) were made to separate proteins in the samples by electrophoresis. 10µl of each sample (approximately 10µg protein per lane) was loaded and run at room temperature in the presence of Running Buffer (50mM Tris HCl, 350mM glycine, 0.2% SDS[w/v]) at constant ampere (15mA/gel) until proteins were well separated. Alongside the samples, 5µl of a molecular weight ladder (Thermoscientific UK, Pageruled Plus Prestained Ladder #26619, range 10-250kDa) was loaded to allow easier identification of proteins by molecular size.

Resolved proteins from the gel were transferred onto nitrocellulose membranes (BioRad UK, #162-0115) in the presence of Transfer buffer (25mM Tris HCl,

200mM glycine, 20% [v/v] Methanol) for 1 hour at 200mA at 4°C. The nitrocellulose membranes were then washed in a plastic box with a protein staining solution (1% [w/v] Amido Black, 5% [v/v] acetic acid, 10% [v/v] Methanol) to confirm good protein transfer and separation. Membranes were then washed repeatedly in water to remove staining.

Membranes were then incubated for 1 hour in Blocking solution (5% powdered milk (w/v), PBS [pH 7.3, 160mM NaCl, 3mM KCl, 8mM Na₂HPO₄, 1mM KH₂PO₄], 0.1% [v/v] TWEEN), with the primary antibodies (PARP, Cell Signalling #9542, 1:1000 and β actin, Abcam UK #8227, 1:5000) then added into the solution. This was incubated overnight at 4°C, then washed 3x 5mins in washing buffer (1x PBS with 0.1% TWEEN), probed with Alexafluor® 750 Goat anti-rabbit secondary antibody (Invitrogen UK, A-21039) at room temperature for 1 hour, washed again in wash buffer 3x 5mins and dried. The fluorescent signal was detected by scanning the membranes with Odyssey Infrared Imaging Scanner by LiCor (LiCor BioSciences Ltd) according to manufacturer's instructions.

4.2.11 Statistical Analysis

Measurements of protein band size were taken using LiCor Odyssey software and the size of the Cleaved PARP (89kDa) band was divided by the size of the β actin band for each lane. The means of 3 independent samples per treatment group was calculated and any statistically significant differences were assessed by one way ANOVA, followed by Bonferroni post hoc tests. All statistical tests were carried out using Graphpad Prism.

4.3. Results

4.3.1 Expression of autophagy-associated genes

Quantitative PCR analysis was used to detect mRNA expression of autophagy genes, *Ulk 1*, *Atg7*, *Atg13* and *Becn1*, following 24 hours of exposure to 0, 5µg/ml cisplatin or 0.2µg/ml doxorubicin. These concentrations were selected as they are the highest used previously and were therefore considered to be the most likely to show an effect. No expression of *Ulk1*, *Atg7* or *Atg 13* was detected. Expression of *Becn1* was detected but no increase was seen following drug-exposure, in fact expression significantly decreased following both cisplatin and doxorubicin (Fig 4.5). These results indicate that autophagy is unlikely to be a key mechanism in the action of cisplatin or doxorubicin. Furthermore, the decrease in *Becn1* expression suggests that apoptosis may be important. Subsequent experiments therefore examined the expression of cleaved PARP, as an indicator of apoptosis.

4.3.2 Cleaved PARP protein expression

Western blots were used to detect cleaved PARP protein expression (representative blots shown in Fig 4.6). A preliminary experiment was undertaken to determine the peak of cleaved PARP expression following cisplatin treatment. This was due to concerns that solely using a single time point of 24 hours of drug exposure could lead to missing the peak of expression of cell death markers. However, results indicate the expression of cleaved PARP is highest at 24 hours over the analysed time period (Fig 4.7). Subsequent experiments therefore used 24 hours of exposure to either cisplatin or doxorubicin. At 24 hours, cisplatin caused a dose-dependant increase in cleaved PARP expression with significant differences detected at 1 and 5µg/ml (Fig 4.8A).

There was a significant increase in cleaved PARP expression following doxorubicin treatment; however this was not as robust an increase as in the cisplatin treated ovaries (Fig 4.8B). Overall, results here indicate that cisplatin and doxorubicin are likely to be causing ovarian cell death through apoptosis but in different patterns of expression.

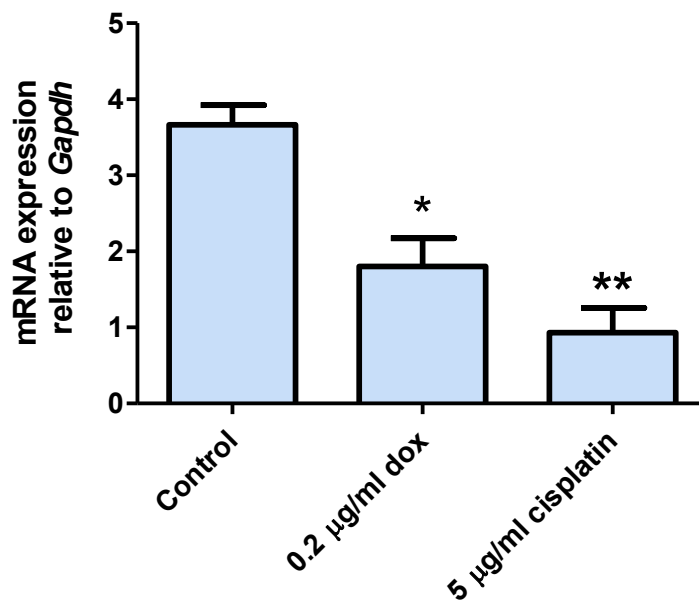


Figure 4.5. mRNA expression of *Becn1* relative to *Gapdh*. Bars denote mean \pm sem, n=3, stars denote significant differences (*p<0.05, **p<0.01).

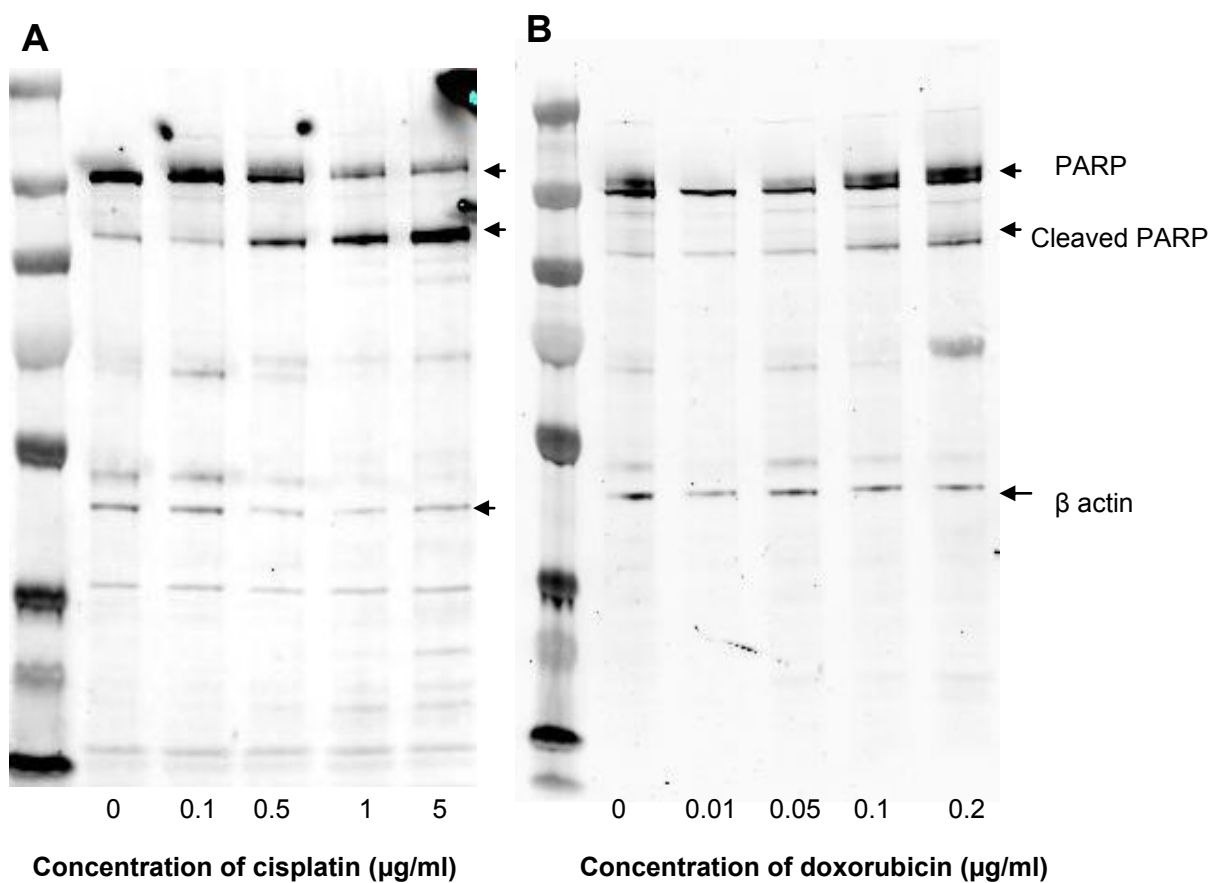


Figure 4.6. Representative Western blots showing Cleaved PARP and β -actin protein expression in ovaries following exposure to increasing concentrations of **A)** cisplatin or **B)** doxorubicin for 24 hours.

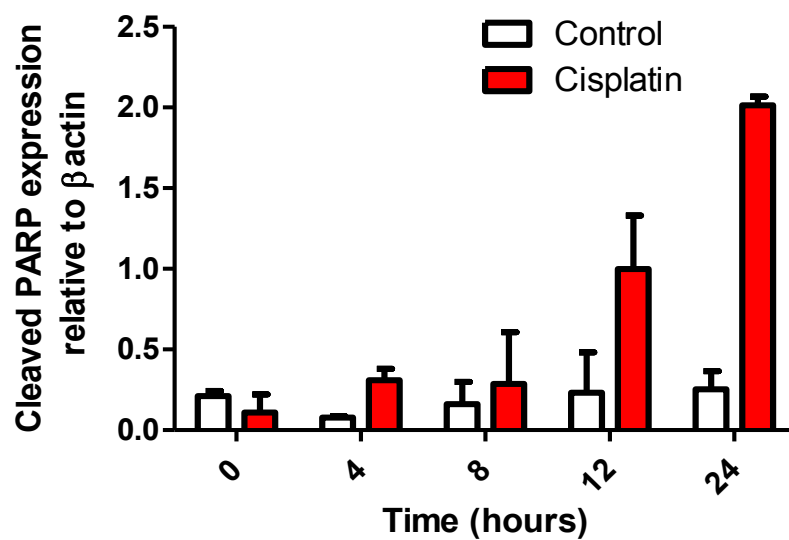


Figure 4.7. Protein expression of cleaved PARP over 24 hours in ovaries treated with cisplatin. Bars denote mean \pm stdev, n=2 for all groups.

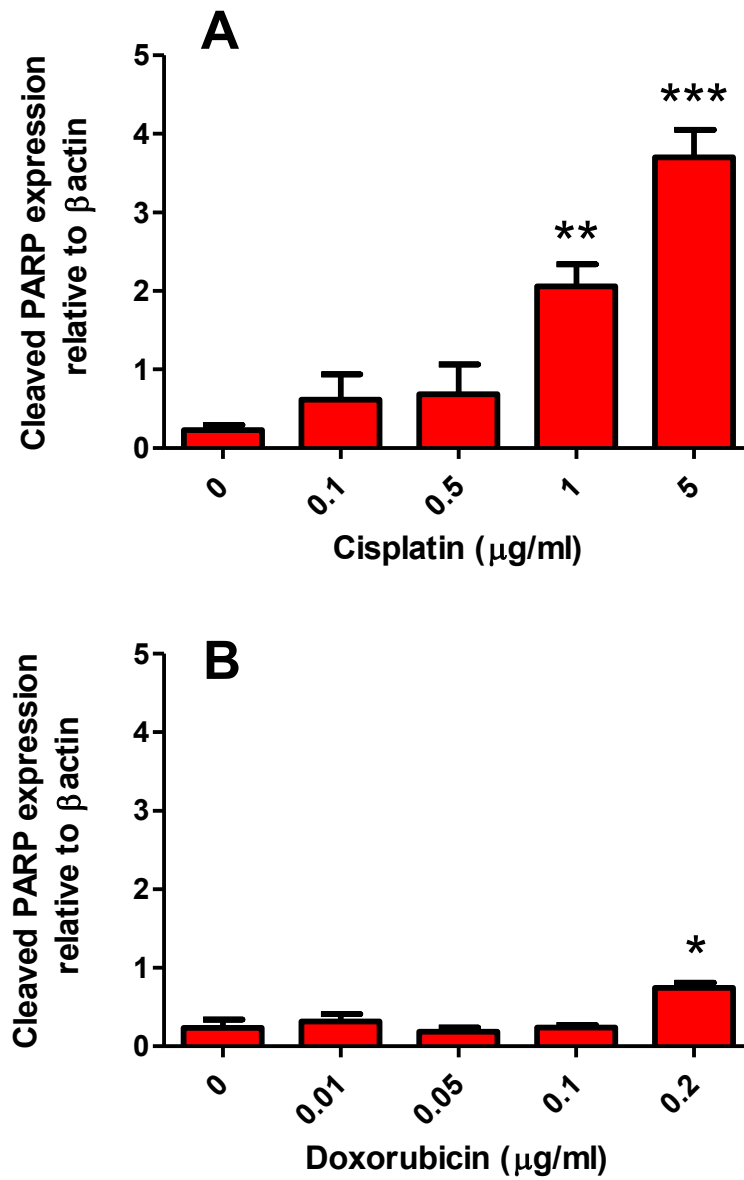


Figure 4.8. Protein expression of cleaved PARP in newborn mouse ovaries treated with **A)** cisplatin or **B)** doxorubicin relative to β actin. Bars denote mean \pm sem, stars denote significant differences relative to control (* p <0.05, ** p <0.01), $n=3$ for each treatment group.

4.4 Discussion

4.4.1 Cell death is unlikely to occur via autophagy

Autophagy is a process of cell death which is being increasingly implicated as having a crucial role in the ovary. No expression of autophagy genes *Ulk1*, *Atg7* or *Atg13* was detected and expression of *Becn1* significantly decreased following drug exposure. Results here therefore indicate that autophagy is not an important component of either cisplatin- or doxorubicin-induced ovarian cell death. There are over 30 genes which have all been identified as being involved in autophagy; the genes selected for analysis here have all been shown to be indispensable for autophagy to occur (Mehrpour *et al* 2010): it is therefore unlikely that autophagy is a major player as none of the genes tested increased in expression.

Autophagy and apoptosis interconnect, with each process negatively regulating the other to produce a balancing act allowing for cell survival. Key to this balance is Beclin1 (Marquez and Xu, 2012), a protein which is essential for autophagy to occur. Beclin1 promotes the formation of the autophagosome, allowing for cytoplasmic components to be sequestered and degraded. Beclin1 can however bind to Bcl-2 family members (Liang *et al*, 1998), which are anti-apoptotic proteins, inhibiting the formation of the autophagosome and thus preventing autophagy. The significant reduction in *Becn1* seen here could therefore be an indication of increased apoptosis (Takacs-Vellai *et al*, 2005). A recent study found that in human melanoma cells, cisplatin downregulated Beclin1 protein expression, with a concurrent increase in the expression of apoptosis markers including caspase 3 (Del Bello *et al*, 2013). A good

extension to this work would be to confirm that the downregulation seen here in *Becn1* mRNA levels also occurs at the protein level, using Western blotting.

No expression of *Ulk1*, *Atg7* and *Atg13* was detected here. The fact that none of these genes were expressed in either control or treated tissue is likely due to too low a level of expression for accurate detection. This is because the genes should be constitutively expressed in this tissue as they are all essential for mammalian autophagy (Mehrpour *et al*, 2010). Pooling more ovaries into each sample could be a way of getting around this problem, as a product was detected at the top of the standard curve for these genes (also indicating that the sensitivity of the assay was the problem rather than primer failure). This would however mean that a very large number of newborn ovaries would have to be pooled to detect a result, which may not be technically that feasible, or to use an amplification kit in order to increase the cDNA concentration in my samples. A better alternative would probably be to investigate protein expression in future, through either Western blotting or immunohistochemistry.

4.4.2 Apoptosis is likely to be involved in cell death

As the expression of *Becn1* indicates that apoptosis may be involved in ovarian cell death following drug exposure, the expression of cleaved PARP was examined. PARP-1 causes ADP-ribosylation of key proteins such as histones and ligases to facilitate DNA repair, an energy dependant process which occurs at the expense of the cells NAD^+ and ATP supply (Burkle and Virag, 2013). The cleavage of PARP-1 is often used as a marker for apoptotic cell death.

There is evidence that PARP can feed into autophagic, necrotic and apoptotic cell death pathways (Shen and Codogno, 2012). Which cell death pathway is activated appears to be dependent on the energy status of the cell, the cell type and the extent to which the cell is injured. As autophagy is induced in situations of cell starvation, a change in energy homeostasis in the cell such as that caused by increased PARP activation could potentially lead to an increase in cell autophagy (Shen and Codogno, 2012). Autophagy appears to be induced by PARP activation as a protective mechanism to aid cell survival (Shen and Codogno, 2012). Necrosis is also an energy dependant process, and so there is evidence that when there is a large increase in the amount of ADP-ribose and subsequent drop in the amount of ATP, cell death by necrosis occurs (De Vos *et al*, 2012). When there is a large amount of DNA damage present, PARP-1 can be cleaved. This is thought to occur in order to prevent the massive energy loss that ensues when PARP is overactivated. This cleavage often occurs through caspase enzymes such as caspase 3 and is therefore considered to be part of the apoptosis mechanism. Although there is some evidence that PARP cleavage is not essential to the process of apoptosis, it is generally considered one of the hallmarks of apoptotic cell death (Koh *et al*, 2005). PARP cleavage can occur during necrosis (Shah *et al*, 1996), however the fragments formed are different sizes to those detected here. There was a large increase in expression of cleaved PARP detected here following the two highest doses of cisplatin treatment, with a much smaller but still significant increase following the highest dose of doxorubicin. This suggests that cisplatin and doxorubicin are causing ovarian cell death through apoptosis although with different patterns of expression for each drug.

It is interesting that TUNEL staining, also considered a hallmark of apoptosis, did not increase significantly in primordial follicles following either drug treatment. It could be that using the time point of 24 hours is not appropriate for TUNEL detection, and that the peak of expression was earlier or later than this time point. A time course experiment would potentially be a good way of checking this. An alternative explanation could be that TUNEL was used to examine primordial follicles, whereas here cleaved PARP was used to detect whole ovary apoptosis; the difference seen may therefore be due to an increase in stromal cell apoptosis which was not quantified in the TUNEL analysis.

4.4.3 Conclusion

Data here shows that apoptosis rather than autophagy is likely to be the mechanism through which cisplatin and doxorubicin cause ovarian cell death. The next step is to try and identify the cell type which is expressing these markers, and compare expression between the two drugs. Work in Chapter 5 will therefore explore cell localization of cleaved PARP and other apoptosis markers.

CHAPTER 5

CELL LOCALISATION OF APOPTOSIS

5.1. Introduction

Apoptosis is a well-defined mechanism of programmed cell death, characterised by distinct morphological changes including cell shrinkage, DNA fragmentation and membrane blebbing. The mechanism of apoptosis is complex, tightly regulated and involves an energy-dependant molecular cascade. One of the earliest studies describing morphological changes in cells consistent with apoptosis (here called chromatolysis) was in 1885, which described cells in rabbit ovarian follicles with fragmented nuclei (Flemming, 1885; Majno and Joris, 1995). The term apoptosis to describe programmed cell death was first used in 1971 in a study which described cells in the liver shrinking and showing chromatin condensation (Kerr, 1971). Since then, apoptosis has been found to be essential during development, as well as cell clearance when cells are considered harmful or senescent. Aberrant apoptosis has been linked to several diseases including cancer and neurodegeneration.

5.1.1 Cleaved PARP

PARP is a DNA repair protein which is cleaved during apoptosis. It is activated in response to DNA damage, allowing for the post-transcriptional modification of proteins essential for DNA repair (Soldani and Scovassi, 2002). If there is large scale DNA damage, PARP is hyperactivated leading to massive depletion of cell energy stores (Boulares *et al*, 1999), and so PARP can be cleaved during apoptosis in order to preserve this energy. Results from Western blotting in Chapter 4 indicated that cisplatin and doxorubicin cause a significant increase in cleaved PARP protein expression in cultured ovaries.

5.1.2 Caspase-3

The most heavily investigated members of the apoptotic signalling pathway are the caspase family of proteins. There have been at least 14 members of the caspase family identified in humans, which are constitutively expressed in virtually all cell types in the body (Kohler *et al*, 2002). They function primarily as proteases, cleaving proteins which are either important for cell function or whose cleavage will initiate further death signalling (Hengartner, 2000). There are many different signals which can initiate caspase-3 mediated apoptosis, with considerable crosstalk between the downstream processes. Disruption of mitochondrial function plays an important role, which includes loss of transmembrane potential and the release of cytochrome *c* into the cytosol (Skulachev, 1998). Once released, cytochrome *c* forms a complex with an adaptor protein Apaf-1 (Apoptotic protease activating factor 1) and caspase-9 (Hengartner, 2000). The activation of caspase 9 allows for the downstream activation/cleavage of other members of the caspase family including caspase-2, 3 and 7 which then cleave downstream target proteins (Fiandalo and Kyprianou, 2012). The mitochondrial pathway is often activated by DNA damage, primarily through upregulation of p53 (Levine *et al*, 2006) which in turn upregulates factors such as Bax. Bax is a pro-apoptotic member of the Bcl-2 family and can directly interact with the mitochondria, to induce changes in membrane potential and hence cytochrome *c* release (Murphy *et al*, 1999). An alternative pathway for caspase-3 activation is the extrinsic binding of a death signal (e.g. Fas) which leads to the activation of caspase-8 (Kaufmann *et al*, 2012) which subsequently cleaves caspase-3. One of the substrates that caspase-3 cleaves is PARP which is also used as a marker of apoptosis. One of the problems with studying caspase family activity is

that there is a large amount of functional redundancy between family members, allowing for different caspase's to compensate for each other's loss.

The best understood caspase in terms of its function in apoptosis is caspase-3. Caspase-3 knockout mice are born at a low frequency, die within a few weeks and they have ectopic masses of cells in the brain indicating a decrease in cell death during brain development (Kuida *et al*, 1996). Caspase-3 appears to be essential for certain aspects of apoptosis to occur, including DNA fragmentation and membrane blebbing as MCF-7 breast carcinoma cells which are lacking functional caspase-3 are killed by inducers of apoptosis but without these morphological changes (Janicke *et al*, 1998). In the ovary, caspase-3 is required for granulosa cell apoptosis during follicle atresia (Matikainen *et al*, 2001). It is expressed in oocytes (Exley *et al*, 1999) but does not appear to be essential for apoptosis during atresia (Matikainen *et al*, 2001).

5.1.3 γ H2AX

H2AX is a histone protein which is randomly distributed throughout the DNA (Altaf *et al*, 2007). When there is a double strand DNA break, H2AX is phosphorylated becoming γ H2AX (Rogakou *et al*, 1998). This phosphorylated protein then acts as a recruiter of DNA repair proteins. Foci of γ H2AX proteins form, which can therefore be used as a sensitive marker for DNA double strand breaks (Pilch *et al*, 2003). Following DNA repair, γ H2AX is dephosphorylated. There is some evidence that double strand DNA breaks are involved in chemotherapy-induced ovarian cell death,

with cyclophosphamide causing a significant increase in oocytes expressing γ H2AX (Petrillo *et al*, 2011).

5.1.4 Aim

Work in the previous chapter indicated that both cisplatin and doxorubicin lead to apoptosis in the ovary. The aim of these experiments was to localise expression of apoptosis markers to different cell types in ovaries treated with cisplatin or doxorubicin.

5.2 Materials and methods

5.2.1 Animals

C57BL6 females were used for all experiments.

5.2.2 Newborn ovary culture

Newborn ovaries were dissected and cultured as described in chapter 2. Ovaries were cultured for 24 hours and then exposed to control medium, cisplatin or doxorubicin for a further 24 hours. As preliminary experiments using Cleaved PARP in immunohistochemistry indicated that the drug dosages used in qPCR and Western analysis in Chapter 4 caused damage too widespread to easily quantify, drug concentrations were reduced. The final concentration of cisplatin used was 0.5µg/ml; the final concentration of doxorubicin was 0.05µg/ml. Ovaries were washed in PBS and fixed immediately after the end of the period of drug exposure. Six ovaries in each treatment group were analysed from three independent cultures.

5.2.3 Histology

Ovaries were fixed in 10% (v/v) buffered formalin (Sigma Aldrich) for approximately 90 minutes then washed in 70% (v/v) ethanol. Formalin was used instead of Bouins for fixing tissue as experiments indicated that Bouins fixative caused non-specific staining following immunohistochemistry. They were then left overnight in 70% (v/v) ethanol with eosin, processed, embedded and sectioned as described in Chapter 2.

5.2.4. Immunohistochemistry using DAB staining

This was used to detect Cleaved PARP (New England Biolabs UK, #9544) and Cleaved Caspase 3 (#9661).

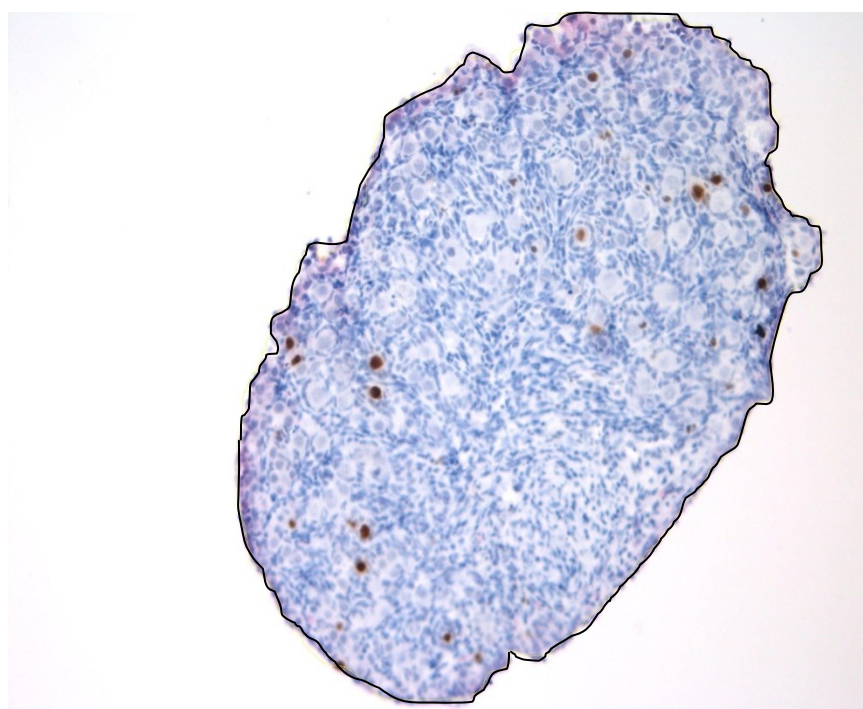
Slides were dewaxed in xylene and rehydrated in decreasing concentrations of ethanol. Slides were then washed in water and boiled in 0.01M sodium citrate buffer for 20 minutes in a microwave, ensuring that slides were always covered in liquid. Slides were cooled before being washed in wash buffer (1xPBS [pH 7.3, 160mM NaCl, 3mM KCl, 8mM Na₂HPO₄, 1mM KH₂PO₄] with 0.1% [v/v] Triton X). They were then incubated for 30 minutes in 3% H₂O₂/90% methanol, to reduce endogenous peroxidase background staining. Slides were then washed in wash buffer and incubated for 1 hour in blocking solution (20% Normal Goat serum/5% bovine serum albumin/PBS). Primary antibodies were then diluted 1:200 in blocking solution and applied to all slides except one, which was the negative control. Slides were incubated in a humidified chamber overnight at 4°C.

Slides were washed in wash buffer and incubated for 1 hour in a 1:200 secondary antibody solution (Goat anti-rabbit biotinylated antibody, Dako UK # E043201-8) diluted in blocking solution. Slides were then washed and incubated for 30mins in ABC kit (Avidin Biotin Kit, Vectastain, #PK6100, Vector Labs UK). Slides were washed in wash buffer and incubated in DAB solution (3,3'-Diaminobenzidine, Vector UK, #SK4100) until brown staining appeared (up to 10 minutes). The reaction was quenched in H₂O, slides were dipped in haematoxylin for 20 seconds, then washed in H₂O. Slides were then placed in STWS (0.2% [w/v] NaHCO₃, 2%

[w/v] MgSO_4 in dH_2O) for 1 minute, washed and then dehydrated in increasing concentrations of ethanol. The ethanol was cleared in xylene for 5 minutes and then slides were mounted with DPX and glass coverslips.

5.2.4.1. Counting and analysis

Two non-adjacent sections more than $50\mu\text{m}$ apart were taken from the middle of each ovary and used for immunohistochemical analysis for each antigen examined. Photomicrographs were taken using a Leica microscope. Sections were analysed blind as to treatment. Any distinct cells which were stained brown were counted and the cell type was identified morphologically. Image J was used to measure the area of each section. The number of brown cells was then adjusted to the area and the average density over the two sections was calculated for each ovary.



$$\text{Area} = 170077.6 \mu\text{m}^2$$

$$170077.6 / 1000000 = 0.170 \text{ mm}^2$$

Number of cleaved PARP +ve cells

Area (mm²)

$$\frac{33}{0.170} = 194.021 \text{ cells/mm}^2$$

Fig 5.1. Example of section analysis of a murine ovary. Image J software was used to draw a line around each section and calculate the area of that section in μm^2 . This was converted to mm^2 and the number of cells counted was divided by this value.

5.2.5 Immunofluorescence

Fluorescence was used for the detection of γH2AX (Abcam UK, #22551), using the Vector Mouse On Mouse kit (Vector, #BMK-2202) according to manufacturers instructions. This kit was chosen due to a high amount of background staining when conventional immunofluorescent staining was used. Slides were dewaxed and antigen retrieved as described in section 5.2.4. They were then incubated for 30 minutes in 3% (v/v) H_2O_2 /90% (v/v) methanol, to reduce endogenous peroxidase background staining which is an extension of that described in the kits instructions. Slides were then washed in wash buffer and incubated for 30 minutes in blocking

solution (Vector labs, M.O.M. blocking solution [2 drops of stock solution to 2.5ml PBS]). Slides were then washed and incubated for 5 minutes in M.O.M. diluent solution (600µl of kit Protein Concentrate stock solution to 7.5ml PBS). This was then tipped off slides and a primary antibody solution was applied (γH2AX, 1:200 in diluent solution) for 30 minutes. Slides were then washed and biotinylated secondary antibody solution (M.O.M. kit) was applied for 10 minutes. Slides were washed and a solution containing streptavidin 488 fluorescent antibody (Alexafluor®, Invitrogen UK, #S11223) diluted 1:200 in diluent solution was applied for 5 minutes, keeping slides in the dark. Slides were then washed, keeping them in the dark, and then a solution of DAPI counterstain (Invitrogen, #D3571, 1:10000 in H₂O) was applied for 20 minutes. Slides were then washed and mounted using Vectashield (Vector, #H-1400) to reduce photobleaching and glass coverslips. Slides were photographed using a Leica A6000 fluorescent microscope.

5.2.6. Statistical Analysis

The number of stained cells was adjusted for area and the average of two sections per mouse ovary was calculated. Six ovaries were analysed like this per treatment group for Cleaved PARP and Cleaved Caspase 3 staining. All data was analysed using Graphpad Prism software. One way ANOVAs determined if significant differences were present across treatment groups, followed by Bonferroni post hoc tests where ANOVA was statistically significant.

5.3. Results

5.3.1 Cleaved PARP

Expression of the apoptosis marker cleaved PARP was analysed in control, cisplatin and doxorubicin treated ovaries, example images of which are shown in Fig. 5.2. Two sections were analysed and averaged per ovary, 6 ovaries were analysed per treatment group. The number of cells expressing cleaved PARP was adjusted for area and compared (Fig 5.3A); there was a significant increase in the number of cleaved PARP positive cells in doxorubicin treated ovaries. The number increased following cisplatin treatment, however this was not significant ($p=0.06$). When cell type affected was broken down into oocytes (Fig 5.3B) or somatic cells (Fig 5.3C), the number of positively stained oocytes did not increase significantly following either drug treatment. There was a significant increase in the number of positive somatic cells following doxorubicin treatment. When somatic cells were broken down further into either granulosa cells (Fig 5.4A) or stroma (Fig 5.4B), there was a significant increase in the number of both cell types staining positive for cleaved PARP.

5.3.2 Cleaved Caspase-3

Expression of cleaved caspase-3 was analysed in control, cisplatin and doxorubicin treated ovaries, example images of which are shown in Fig. 5.5. Two sections were analysed and averaged per ovary, 5 ovaries were analysed per treatment group. When the number of cells which stained positive for cleaved caspase 3 was analysed, there was no significant increase following either cisplatin or doxorubicin treatment (Fig 5.6A). When cell type affected was broken down into oocytes (Fig 5.6B) or somatic cells (Fig 5.6C), there was a non-significant increase in the number of positive

oocytes following cisplatin treatment. When somatic cells were broken down further into either granulosa cells (Fig 5.7A) or stroma cells (Fig 5.7B), there was a significant increase in the number of granulosa cells stained positive for cleaved caspase-3 following doxorubicin treatment. In contrast to cleaved PARP analysis, there was no significant increase in stromal cell expression.

Analysis of the stromal cells was more challenging than with cleaved PARP, with some vessel-like structures identified with positive cleaved caspase-3 staining; when these were counted there was a significant increase in the number following doxorubicin treatment when compared to control (Fig 5.8). As these have a similar phenotype to blood vessels, double immunofluorescence was used to see if there was co-localisation of cleaved caspase-3 with CD31, an endothelial marker. There were many problems optimising the experimental protocol for this and it was not sufficiently optimised in time.

5.3.3 γ H2AX

Sections from ovaries treated with control, cisplatin and doxorubicin were stained for γ H2AX (Fig 5.9). Etoposide-treated ovaries (material supplied by Agnes Stefansdottir) were used as a positive control, as it is well established that etoposide causes double strand DNA breaks (Smart *et al*, 2008). There were a lot of problems with high background staining in preliminary experiments, possibly due to the fact that the primary antibody was raised in mouse. These were overcome to some extent through use of a Mouse on Mouse blocking kit (Vector), however due to these

technical problems, there was not time for a detailed analysis of γ H2AX expression in the cisplatin- or doxorubicin- treated ovaries.

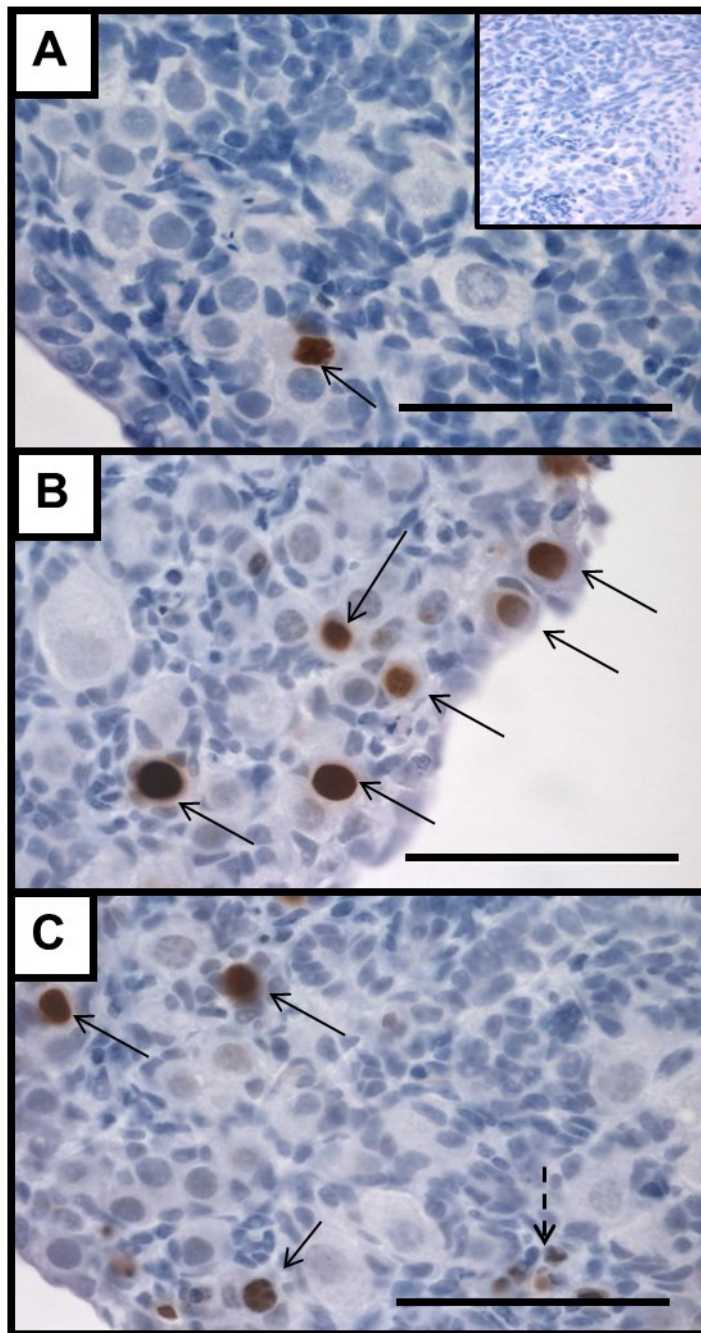


Figure 5.2. Representative immunohistochemistry images of cleaved PARP expression in **A)** control, **B)** cisplatin or **C)** doxorubicin treated mouse ovaries. Inset shows negative control. Solid line arrows show positively stained oocytes as determined by their morphology. Dashed arrow shows a cluster of stained stromal cells. Scale bar represents 50µm.

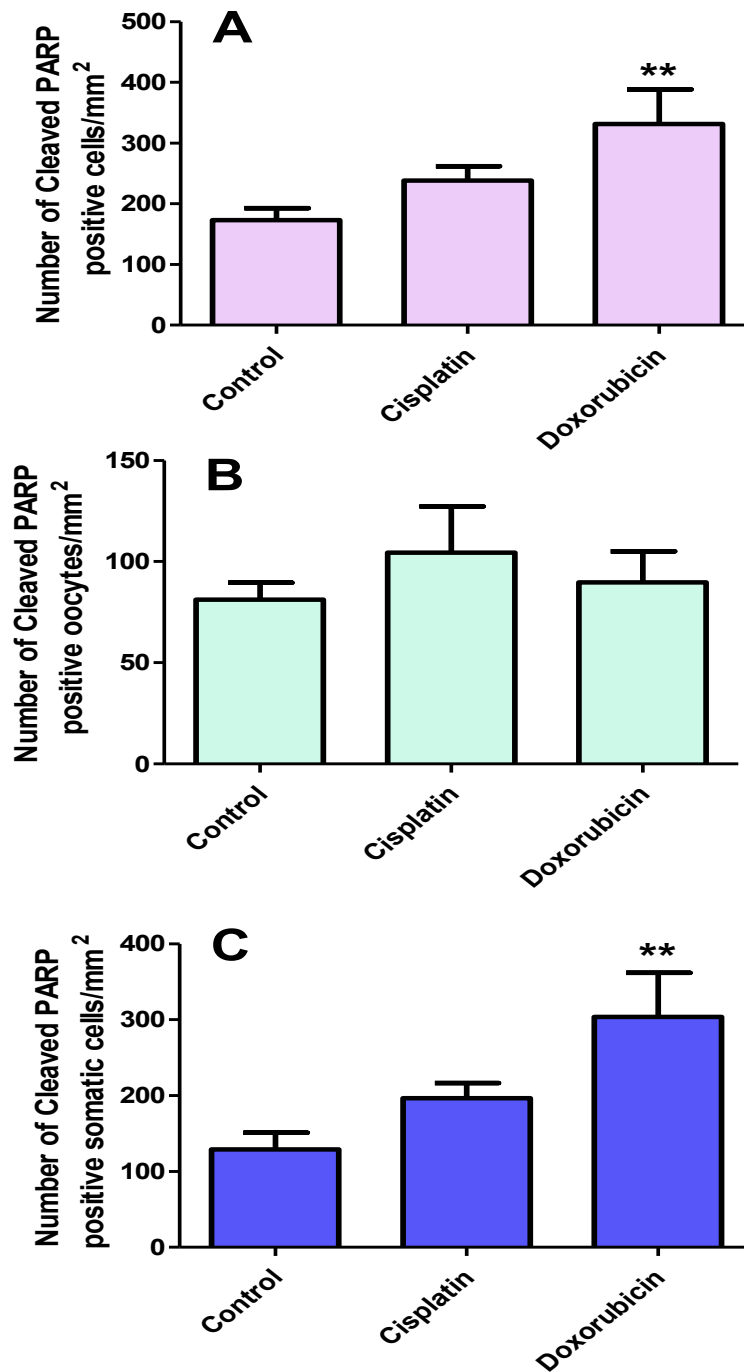


Figure 5.3. Number of cleaved PARP positive **A)** cells, **B)** oocytes and **C)** somatic cells in ovaries treated with 0.5µg/ml cisplatin or 0.05µg/ml doxorubicin. Bars denote mean±sem, n=6 for each treatment, stars denote significant differences (**p<0.01).

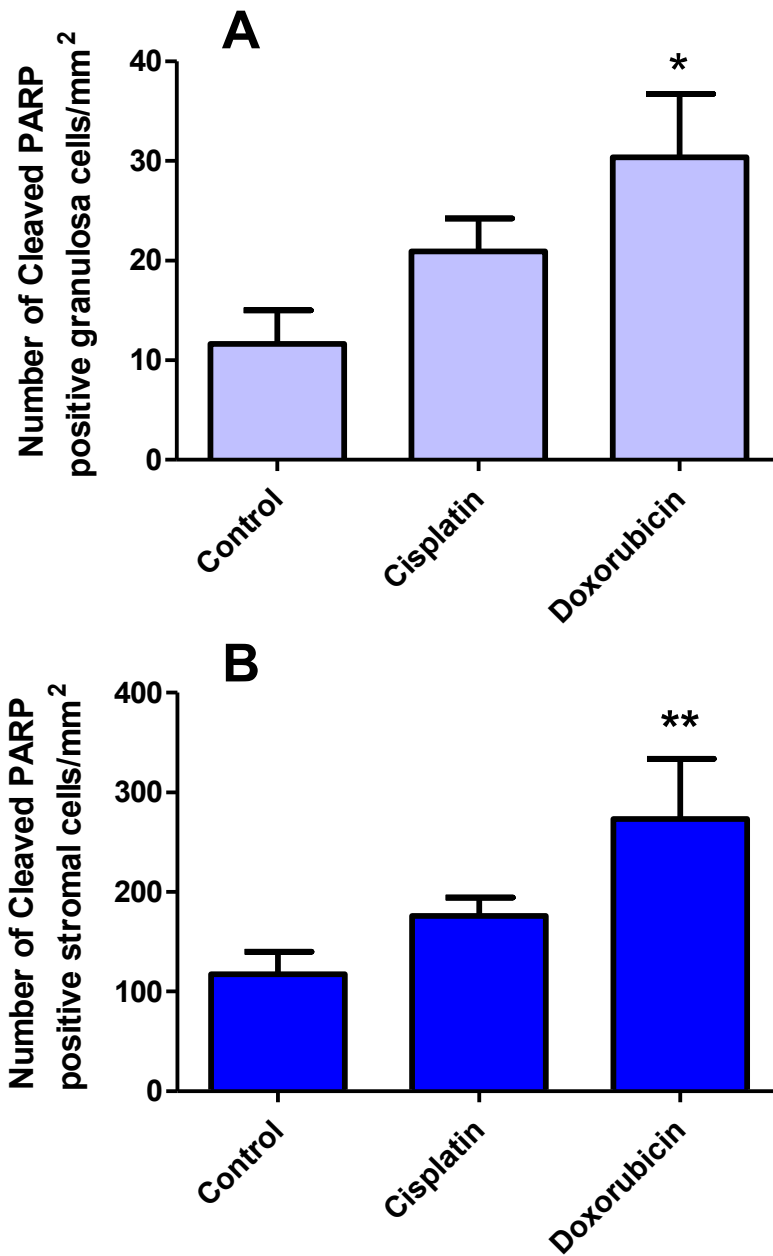


Figure 5.4. Number of cleaved PARP positive **A)** granulosa cells and **B)** stromal cells in ovaries treated with 0.5 μ g/ml cisplatin or 0.05 μ g/ml doxorubicin. Bars denote mean \pm sem, n=6 for each treatment, stars denote significant differences (*p<0.05, **p<0.01).

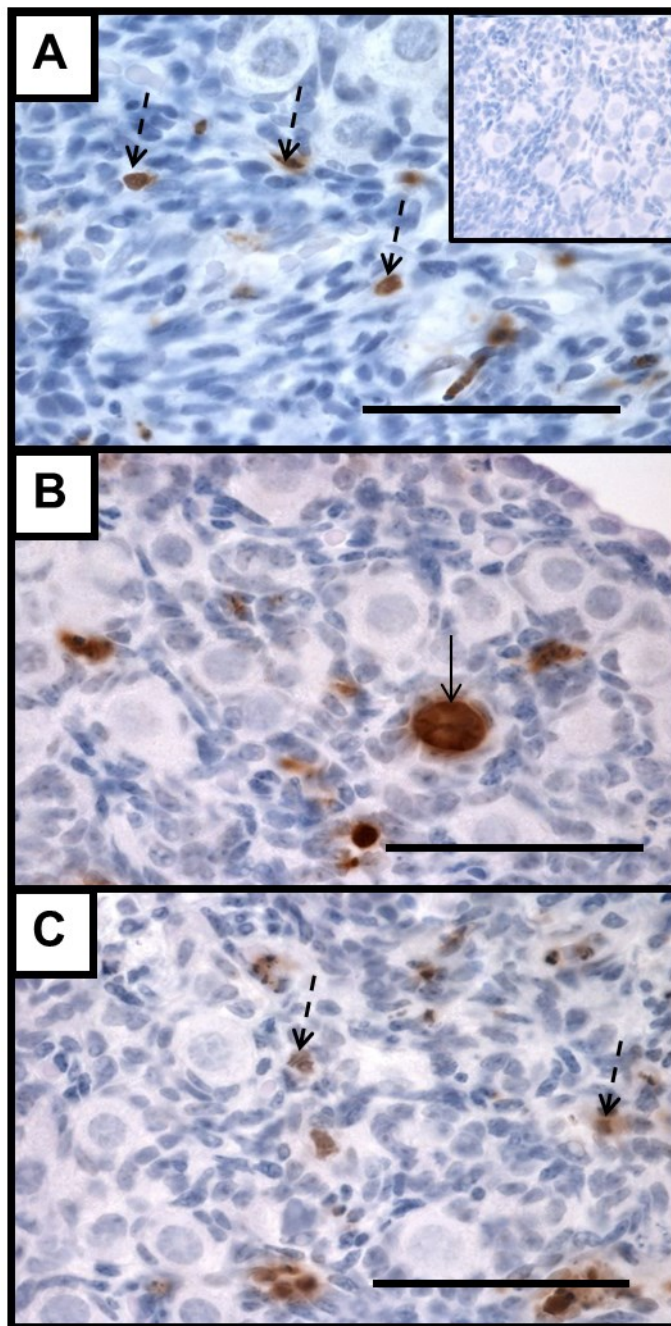


Figure 5.5. Representative immunohistochemistry images of cleaved caspase-3 expression in **A)** control, **B)** cisplatin or **C)** doxorubicin treated newborn mouse ovaries. Inset shows negative control. Solid line arrows show positively stained oocyte as determined by its morphology. Dashed arrows show stained stromal cells. Scale bar represents 50 μ m.

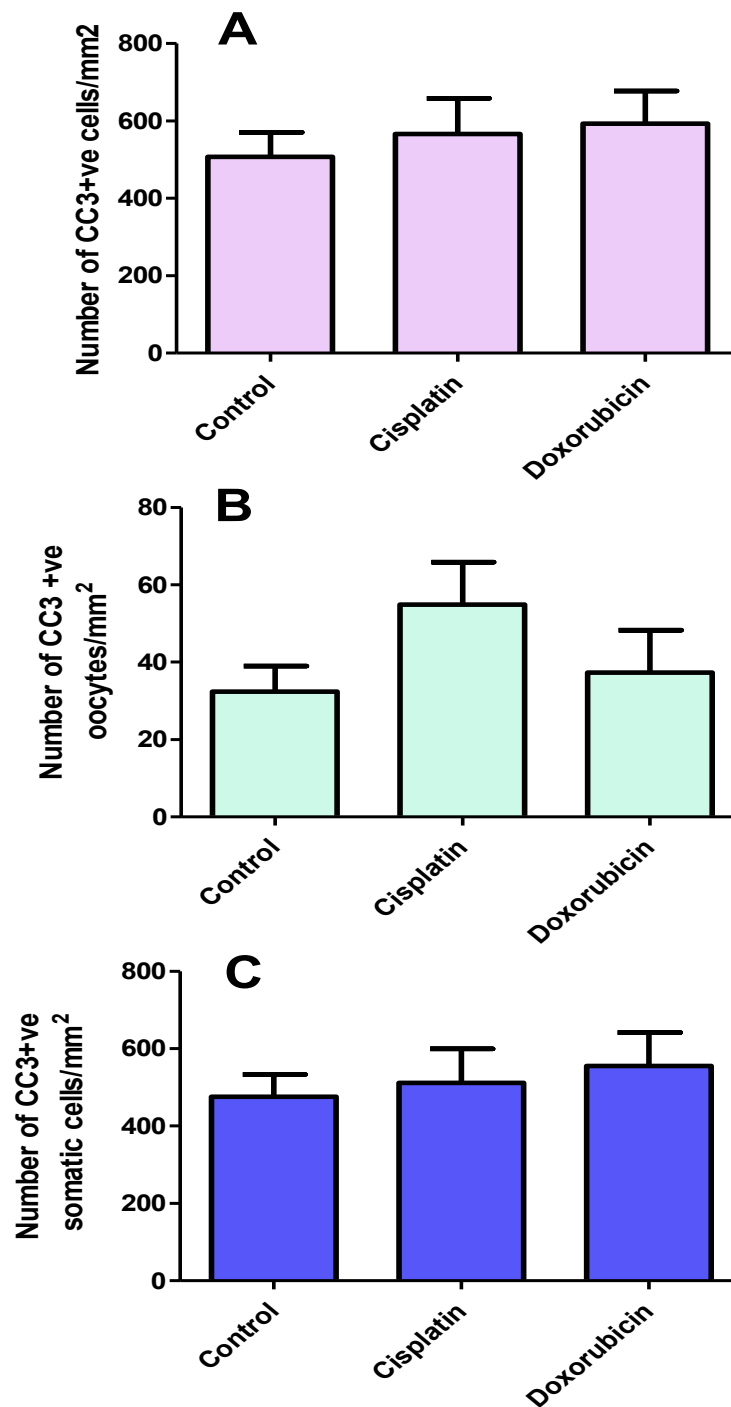


Figure 5.6. Number of cleaved caspase 3 positive **A)** cells, **B)** oocytes and **C)** somatic cells in newborn mouse ovaries treated with 0.5 μ g/ml cisplatin or 0.05 μ g/ml doxorubicin. Bars denote mean \pm sem, n=5 for each treatment.

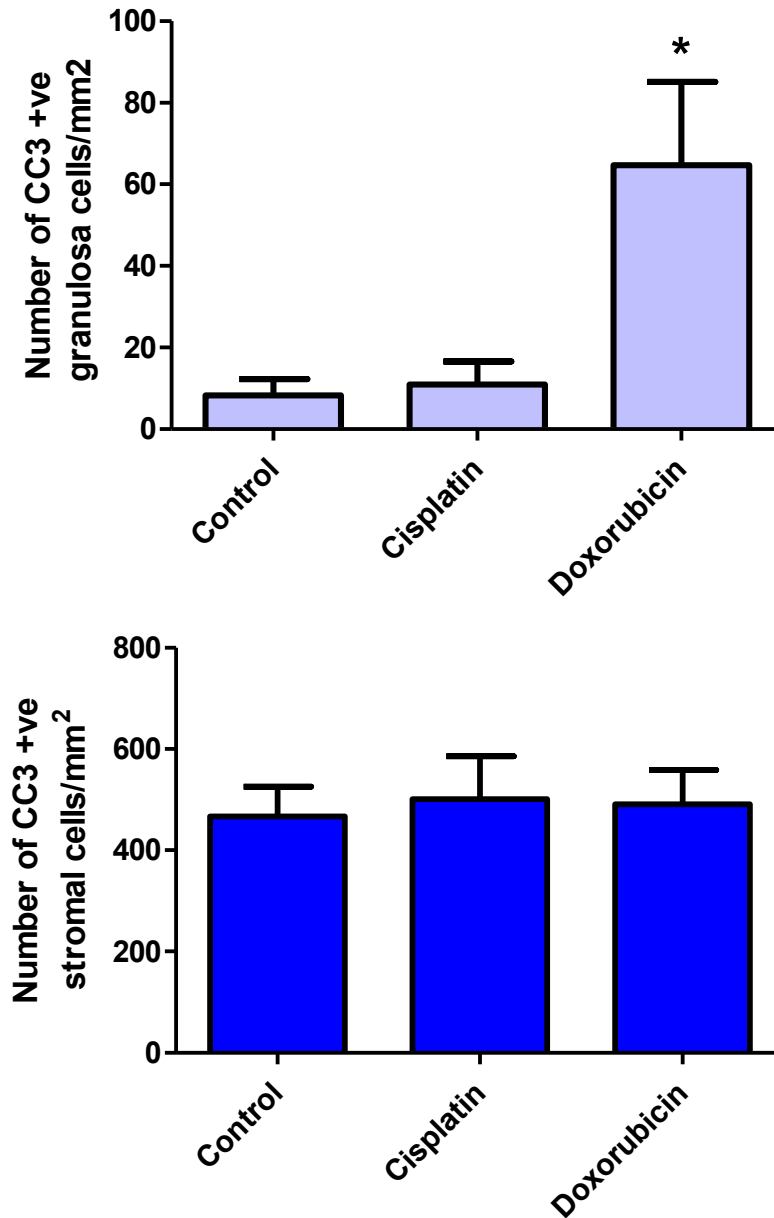


Figure 5.7. Number of cleaved caspase 3 positive **A)** granulosa cells and **B)** stromal cells in newborn mouse ovaries treated with 0, 0.5µg/ml cisplatin or 0.05µg/ml doxorubicin. Bars denote mean±sem, n=5 for each treatment, stars denote significant differences relative to control (*p<0.05, **p<0.01).

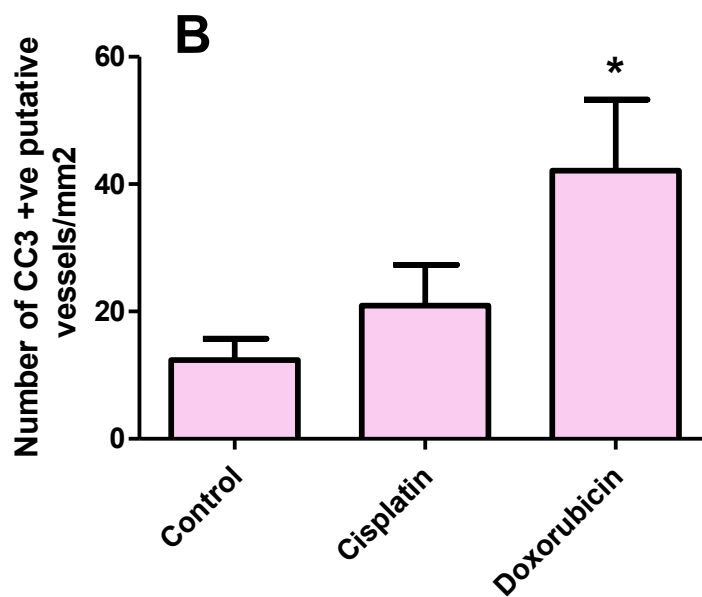
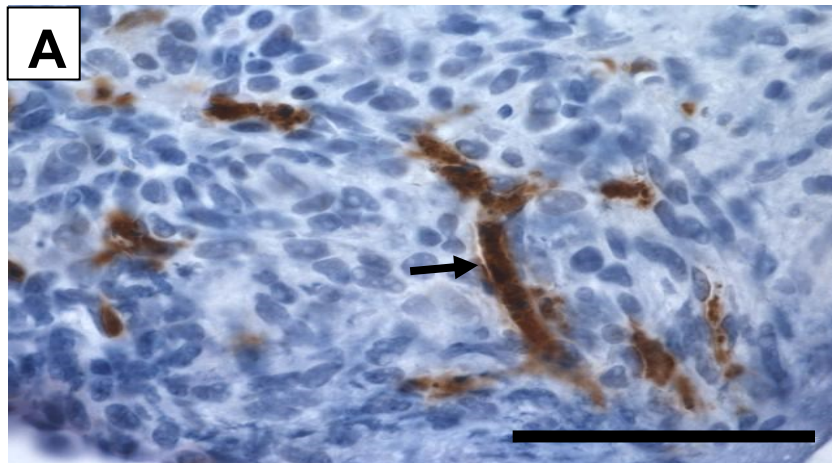


Figure 5.8. A) Example of Cleaved caspase 3 expression in the stroma which resembles a vessel as highlighted by the black arrow (structure classified as single vessel), **B)** number of possible vessels stained positive for cleaved caspase 3 in ovaries treated with 0, 0.5 μ g/ml cisplatin or 0.05 μ g/ml doxorubicin. Bars denote mean \pm sem, n=5 for each treatment, stars denote significant differences (*p<0.05).

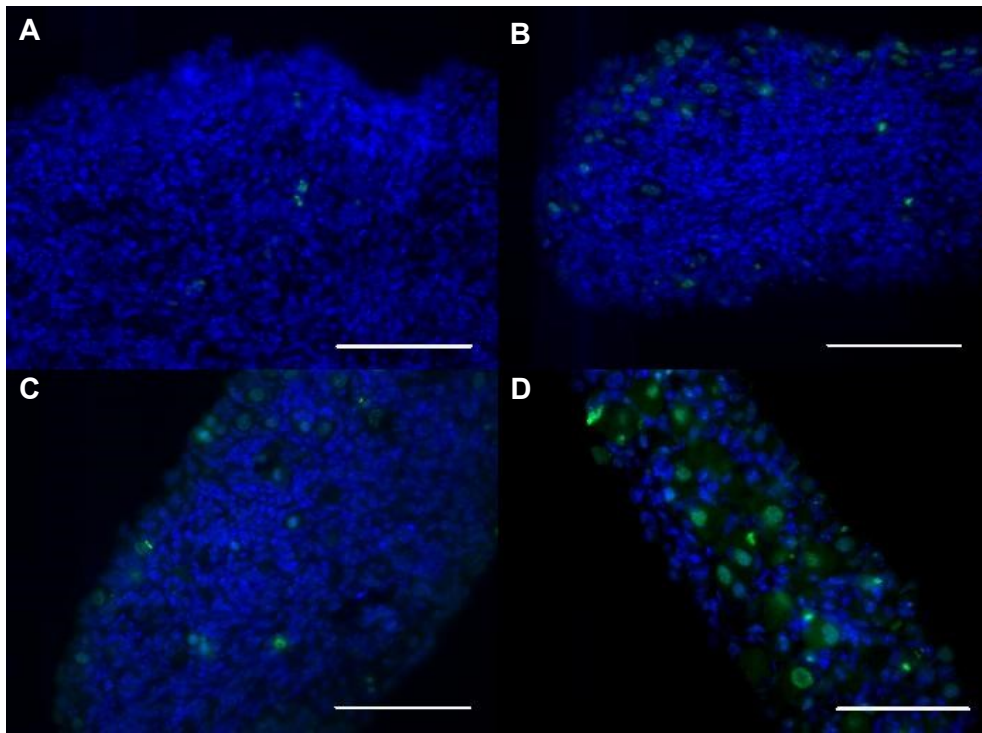


Figure 5.9. Expression of γ H2AX (green) in newborn mouse ovaries (nuclei stained blue) treated with (A) control, (B) cisplatin, (C) doxorubicin and (D) etoposide which was used as a positive control. Scale bar represents 50 μ m.

5.4 Discussion

Apoptosis markers cleaved PARP, cleaved caspase-3 and γ H2AX, were used to determine cell localisation of apoptosis in newborn mouse ovaries cultured with cisplatin or doxorubicin. Results here suggest that both drugs induce apoptosis, with different patterns of cellular expression. Doxorubicin causes apoptosis in ovarian somatic cells; cisplatin's action is less clear.

5.4.1 Cisplatin

There was no significant increase in the number of cells expressing cleaved PARP or cleaved caspase-3 following cisplatin treatment. However, the near-to-significant p value for cleaved PARP analysis ($p=0.06$) could well indicate that this could become significant if the sample size was increased. Clearly, increasing the n number to determine if this is the case or not, is important future work. Although the results were not significant and so no firm conclusions can be drawn, there were some interesting trends. The increase in the number of oocytes positive for cleaved caspase-3 in particular, although not significant, is notable and so extending the study with a larger sample size could yield a more significant result. Alternatively, the concentration of cisplatin used could be slightly increased. Cell localisation of apoptosis markers TUNEL and γ H2AX in another study following cisplatin treatment indicated that cisplatin targets the oocyte; there was a much higher concentration of cisplatin used in this study than used here ($6\mu\text{g/ml}$ compared to $0.5\mu\text{g/ml}$ used here) (Gonfloni *et al*, 2009). Therefore, increasing the concentration of cisplatin used could potentially lead to more significant results. Nonetheless, the concentrations used here are in serum range (Pfeifle *et al*, 1985; Urien and Lokiec,

2004), which causes damage and follicle loss and so it could be that using a different time point or an alternative method of detection will yield significant results.

There is a discrepancy between results in Chapter 4 and here, with Chapter 4 demonstrating a large scale, robust increase in cleaved PARP expression following cisplatin treatment. This increase was however only significant at 1 and 5µg/ml cisplatin and so again, the smaller concentration which was used here may account for this difference.

5.4.2 Doxorubicin

Doxorubicin induced a significant increase in the number of granulosa cells which express the apoptosis markers cleaved PARP and cleaved caspase-3. This corroborates results in Chapter 3, which saw a significant increase in the percentage of follicles with pyknotic granulosa cells. This suggests that granulosa cells are particularly sensitive to doxorubicin treatment and is consistent with other reports (Ben-Aharon *et al*, 2010; Roti Roti *et al*, 2012). The reason that granulosa cells are so sensitive to doxorubicin could be related to the fact that they are so mitotically active; doxorubicin interferes with cellular replication and transcription. This damage to the granulosa cells could cause indirect germ cell death and hence follicle loss.

There was a significant increase in the number of cleaved PARP positive stromal cells following doxorubicin, indicating that doxorubicin also causes apoptosis in the stroma. Most studies which have investigated the effect of chemotherapy on the ovary concentrate on direct follicle action; few describe stromal specific effects. One

ultrastructural study of ovarian biopsies in girls who underwent treatment for leukaemia found moderate to severe signs of stromal damage as well as capillary changes (Marcello *et al*, 1990). A recent study investigating the action of doxorubicin on the ovary found that cell death (as analysed by neutral comet assay which detects DNA breaks) appeared first in the stroma, with expression appearing in the granulosa cells, then the oocytes over time (Roti Roti *et al*, 2012). Toxic insult to the stromal cells could have detrimental effects on the follicles in the ovary and be an indirect method through which follicle loss could occur.

The ‘vessel’ structures observed also showed significantly increased expression of cleaved caspase-3 following doxorubicin treatment. Their morphology suggests that they could be blood vessels; unfortunately this could not be confirmed due to technical difficulties. Vasculature changes have been suggested as an additional mechanism by which chemotherapy could cause follicle loss through local ischemia (Meirow *et al*, 2007). The morphology of these ‘vessels’ is similar to that described in a study which used doxorubicin in human cortical pieces (Soleimani *et al*, 2011). There, immunostaining for the endothelial marker CD31 found a significant reduction in vessel density following doxorubicin treatment.

It is notable however that these structures were identified here with cleaved caspase-3 staining only and not with cleaved PARP. It could be that these are some sort of artefact of this antibody’s staining. This may also explain why there is no significant difference in positive stromal cells following cleaved caspase-3 in contrast to cleaved

PARP; as the staining was less distinct, the counting of positive stromal cells may have been less accurate than in the cleaved PARP analysis.

The difference in expression of cleaved PARP and cleaved caspase-3 is intriguing, as they are both used to identify apoptosis. As well as antibody-related staining problems, there are other possible explanations for this. Apoptosis is a molecular cascade, involving signals which are expressed transiently. This could mean that the difference seen here may be related to the fact that a single time point of 24 hours was used for analysis.

5.4.3 γ H2AX

γ H2AX is a marker for DNA double strand breaks (Pilch *et al* 2003). Although, there was not time to perform a full analysis, preliminary results are interesting and worth pursuing. In both cisplatin and doxorubicin treatment, there appears to be a slight increase in cells (predominantly oocytes) with γ H2AX foci. As these images are fluorescent, it is slightly more difficult to determine cell type based solely on their morphology. It would therefore be worth immunostaining for γ H2AX in conjunction with a cell specific marker in future (e.g. vasa/MVH [Mammalian vasa homologue] for oocytes [Castrillon *et al* 2000]). There also appears to be a huge increase in expression of γ H2AX in the etoposide treated ovaries when compared to the other treatment groups. This is expected, as etoposide is a well-known inducer of DNA breaks (Tanaka *et al* 2007). What is perhaps more surprising is the difference in expression following etoposide treatment when compared to cisplatin or doxorubicin. Although these results are preliminary, this may suggest that DNA double strand

breaks are not actually that important a mechanism of cisplatin or doxorubicin-induced cell death. Cisplatin has been shown previously to induce an increase of γ H2AX foci in oocytes, however this was following a much higher dosage of cisplatin (6 μ g/ml compared to 0.5 μ g/ml used here) (Gonfloni *et al*, 2009). In primary granulosa cell cultures, doxorubicin induces DNA double strand breaks as assessed by comet assay (Roti Roti and Salih, 2012). In the whole ovary, doxorubicin in another study caused an increase in γ H2AX expression in primordial follicles following 24 hours of exposure; however the dosage used here was high (20mg/kg) (Roti Roti *et al*, 2012). To put this dosage into perspective, rats treated with 6mg/kg of doxorubicin have a peak plasma concentration of doxorubicin of 1.7 μ g/ml (Rahman *et al*, 1986), the ovaries here were exposed to 0.05 μ g/ml which is in the human serum range (Greene *et al*, 1983). It could therefore be that the other studies are detecting an increase in DNA double strand breaks following these drugs because they are using higher, more pharmacological concentrations of cisplatin or doxorubicin.

5.4.4. Future Work

As well as completing the work described above, there are other directions which this research could take. Now that it has been determined that apoptosis is likely to be the key mechanism through which these drugs are causing ovarian cell death and the differences in cellular localisation, it is important to consider why these different cells are more vulnerable to each drug. Finding how the drugs initiate apoptosis in each cell type will potentially allow for treatments to be designed that can block

these cell effects. Potential pharmacological treatments for preventing ovarian damage will be discussed further in Chapter 6.

5.4.5 Conclusion

Cell localisation of apoptosis induced by cisplatin and doxorubicin in the ovary was investigated. Results here show that doxorubicin causes apoptosis in the somatic cells of the ovary, particularly in the granulosa cells. Cisplatin's action and cell localisation is less clear.

CHAPTER 6

**THE ABILITY OF IMATINIB MESYLATE TO
PROTECT THE OVARY FROM CISPLATIN- OR
DOXORUBICIN-INDUCED DAMAGE**

6.1 Introduction

Due to the increasing number of women affected by POF as a result of chemotherapy treatment, preservation of fertility (now termed oncofertility) is a question of growing importance. The established options currently available to women and girls for fertility preservation include cryopreservation of oocytes, ovarian tissue and embryos for women with life partners (Wallace *et al*, 2005). The ideal solution would be if the ovary could be directly protected from any toxic effects of chemotherapy drugs, thus preserving fertility and alleviating symptoms which are associated with premature menopause.

6.1.1. Current options to protect fertility

6.1.1.1 Cryopreservation

The most reliable method of preserving fertility remains the cryopreservation of embryos as embryos are less vulnerable to cryopreservation-induced damage than oocytes (Blumenfeld *et al*, 2008). There are, however, several disadvantages to this option: it is not an option for very young patients who do not ovulate or do not have a partner, for some older patients it will be reliant on a sperm donor (which can present legal and ethical issues) and for all it requires time for an IVF cycle which the patient may not have (Tao and Valle, 2008). There is also the concern that IVF may be detrimental for patients who have estrogen-sensitive tumours. Oocyte cryopreservation has resulted in pregnancies (Kuleschova *et al*, 1999; Selvaraj *et al*, 2009) but overall this technique is still fairly experimental (Qiao *et al*, 2013). Cryopreservation of ovarian cortex and its reimplantation following treatment is an option available to females of all ages. For the young patient, cryopreservation of

ovarian cortical tissue collected laparoscopically is extremely promising with the birth of twenty live infants reported so far following reimplantation of frozen-thawed tissue (Donnez *et al*, 2012). Ovarian tissue cryopreservation has the potential advantages of preservation of a large number of oocytes within primordial follicles, it does not require hormonal stimulation when time is short, and is appropriate for the pre-pubertal girl. Disadvantages include the need for an invasive procedure, and the uncertain risk of ovarian contamination in hematological and other malignancies (Rosendahl *et al*, 2010b; Shaw *et al*, 1996). As well as the small size of the tissue, reimplantation following cryopreservation or vitrification causes substantial follicle loss (Abir *et al*, 2011) which also limits the lifespan of the tissue and so can never replace long term fertility or protection from symptoms of the menopause.

6.1.1.2 GnRH agonists/antagonists

Although the effectiveness of administering GnRH agonists to prevent chemotherapy induced ovarian damage has yet be fully proven, this is, in places, a widely used clinical treatment. These agonists are used to suppress the hypothalamic-pituitary-gonadal axis, thereby returning the ovary to a more quiescent state (Sonmezer and Oktay, 2004). The hypothesis that this suppression could protect the ovary is based on observations that prepubertal girls appear less susceptible than older women to the damaging effects of chemotherapy on fertility (Ortin *et al* 1990). However, these observations could be a result of the larger follicle reserve in prepubertal patients, as well as difficulties in assessing the follicle reserve in young patients (Brougham *et al* 2012). Reduction of the follicle reserve in these patients will also manifest many

years after therapy, which may account for the difference between these and older women, rather than the prepubertal environment being in itself protective.

The mechanism by which GnRH agonists are thought to protect the ovary is unclear. One hypothesis is that it restricts blood flow to the ovary, allowing less of the chemotherapeutic agent access to the ovarian reserve. A rat model system has shown that following GnRH agonist treatment, there is a decrease in ovarian vascular permeability and density (Kitajima *et al*, 2006), however the relevance of ovarian blood flow to human physiology remains disputed. It has also been hypothesised that GnRH agonists could act directly on the ovary. This seems unlikely to be responsible for a long term protective effect, as GnRH receptors are only present in preovulatory follicles and the corpus luteum (Choi *et al*, 2006), neither of which would be present in the quiescent ovary. GnRH agonists have a protective effect against doxorubicin directly on granulosa cells *in vitro* (Imai *et al*, 2007) but these granulosa cells were taken from mature follicles which express GnRH receptors. GnRH agonists have been shown to cause an inhibition of follicular recruitment (Ataya *et al*, 1989), however any direct effect of them on smaller follicle classes remains unclear.

There have been several clinical studies investigating the efficacy of GnRH agonists in protecting the ovarian reserve, with conflicting conclusions being drawn as many such studies are not randomised, have small patient numbers, occasionally lack controls and are often case studies (Beck-Fruchter *et al*, 2008). Two large-scale randomised multicentre clinical trials were published in 2011, but unfortunately these had conflicting results. In the PROMISE-GIM6 study, a significant reduction in

chemotherapy-induced ovarian failure was found following GnRH agonist administration (Del Mastro *et al*, 2011). However, following GnRH treatment, the GBG 37 ZORO study did not find a statistically significant reduction in chemotherapy-induced POF (Gerber *et al*, 2011). While the outcome measures of both studies were primarily menstrual function with limited endocrine analysis, it is unclear why different results were found. Both assessed POF after a relatively short time (PROMISE-GIM6 study after one year; the ZORO study after six months) and there remains a need for long term follow up, to fully determine whether GnRH agonists are having a lasting protective effect on the ovary.

6.1.2 Potential protective treatments

Directly protecting the ovary from damage induced by chemotherapeutic agents would be the ideal method through which fertility could be preserved. There are several key requirements which this potential protective treatment would have to fulfill. It could not interfere with the anti-cancer action of the chemotherapy agent it was protecting against. It would also have to be non-toxic in itself, both to the ovary and to the patient in general. Finally it would have to have no negative effects on the competence of the oocytes it was protecting. Several potential treatments currently under investigation are discussed below.

6.1.2.1 Ceramide

Ceramide is a sphingosine-based lipid signalling molecule which, when produced in high levels in cells, can lead to apoptosis (Morita and Tilly, 2000). When the ceramide pathway is manipulated by adding sphingosine-1-phosphate (S1P), a

downstream antiapoptotic metabolite of ceramide, mature oocytes are protected *in vitro* from doxorubicin-induced cell death (Perez *et al*, 1997). Rodents treated *in vivo* with SIP have a decrease in primordial follicle death following chemotherapy exposure (Hancke *et al*, 2007) and there is no discernible genomic damage in the offspring of rodents given such ovarian protection against irradiation (Paris *et al*, 2002). Primates which were exposed to radiation alongside an SIP analogue demonstrated a protective effect on ovarian function (Zelinski *et al*, 2011); whether this can be replicated in humans or following chemotherapy treatment remains to be seen.

6.1.2.2 AS101

AS101 (ammonium tri-chloro(dioxoethylene-O,O')tellurate) was developed as a potential immunomodulatory treatment (Sredni *et al*, 1987). It has been shown to protect against hematopoietic damage and alopecia induced by cyclophosphamide, carboplatin and etoposide (Kalechman *et al*, 1991; Sredni *et al*, 1995). AS101 appears to function through stimulating the secretion of cytokines such as IL-1 and the inhibition of IL-10 (Hayun *et al*, 2007). IL-10 is essential for tumour cell proliferation, meaning that AS101 has anti-tumour properties in itself as well as increasing the efficacy of chemotherapy agents used in conjunction with it (Sredni *et al*, 2004). IL-10 inhibition also leads to a downstream activation of Akt, a serine-threonine kinase which has been shown to be an important suppresser of male germ cell apoptosis following radiotherapy (Rasoulpour *et al*, 2006). AS101 has been used in conjunction with cyclophosphamide in male mice and it was found that AS101 caused a significant reduction in sperm DNA fragmentation and seminiferous tubule

damage as well as a partial rescue of fertility when compared to cyclophosphamide alone (Carmely *et al*, 2009). A recent study in the ovary suggests that AS101 can protect against cyclophosphamide-induced follicle loss, primarily through reducing primordial follicle activation (Kalich-Philosph *et al*, 2013).

6.1.2.3 Dexrazoxane

Dexrazoxane is a chemoprotectant which has been investigated as a method for blocking doxorubicin-induced cardiotoxicity (Swain *et al*, 1997) and is currently approved for clinical use as such. There is also some evidence that dexrazoxane may be effective against etoposide-induced toxicity in bone marrow and nervous system tumours (Attia *et al*, 2009; Hofland *et al*, 2005). There are two mechanisms through which dexrazoxane can potentially act. The first is through an interaction with topoisomerase II enzyme. Doxorubicin interacts with topoisomerase II, allowing it to bind to DNA but preventing it from resealing DNA breaks (Burden and Osheroff, 1998). Dexrazoxane binds to a different site on the topoisomerase enzyme and prevents it from binding onto DNA altogether (Hasinoff and Herman, 2007), whether doxorubicin is present or not. This means that doxorubicin is unable to induce DNA damage. The other mechanism by which dexrazoxane may work is that it is metabolised in the cell to an EDTA derivative. This derivative chelates iron which could reduce oxidative stress (Vile and Winterbourn, 1990). Importantly, dexrazoxane use in conjunction with doxorubicin does not prevent the anti-tumour activity of doxorubicin in breast cancer (Marty *et al*, 2005) nor does it lead to secondary malignancies in pediatric cases of leukaemia (Barry *et al*, 2008). One study so far has used dexrazoxane on granulosa cell cultures and found a reduction in

DNA damage with co-treatment when compared to doxorubicin alone (Roti Roti and Salih, 2012). However, another study has found dexrazoxane treatment alone causes increased germ cell apoptosis in foetal human ovaries (Poulain *et al*, 2012). The effectiveness of this treatment on preserving the follicle reserve in whole postnatal ovaries or the subsequent viability of the germ cells which do remain is yet to be shown.

6.1.2.4. Tamoxifen

Tamoxifen is a chemotherapeutic agent introduced in the 1970s and is primarily used for the treatment of estrogen receptor positive breast cancer (Jordan, 2003). It functions as a selective oestrogen receptor modulator and is anti-estrogenic in mammary tissue. As it is extensively used clinically both alone and in combination with other chemotherapy agents, it would be an ideal protective agent as its safety has already been established. In rats treated with cyclophosphamide, there was a decrease in primordial and total follicle number, an effect which was rescued following co-treatment with tamoxifen (Ting and Petroff, 2010). Tamoxifen also reversed the detrimental effect of cyclophosphamide on neonatal survival in this study (Ting and Petroff, 2010). A randomized trial which followed women one year after CMF (Cyclophosphamide, methotrexate and 5-fluouracil) treatment for breast cancer found no significant increase in the percentage of menstruating women following tamoxifen co-treatment (n=60) when compared to CMF alone (n=63) (Sverrisdottir *et al*, 2009). The efficacy of tamoxifen as a means to protect the ovarian reserve from chemotherapy-induced induced damage is therefore not yet clear.

6.1.2.5. Amifostine

Amifostine, also known as Ethyol or WR-2721, is a thiol prodrug which was designed to function as a protective agent in normal tissue against radiation-induced damage (Kouvaris *et al*, 2007). It functions primarily as a scavenger of free radicals and is selective, as it concentrates more rapidly in normal cells than tumours (Citrin *et al*, 2010). It is also considered to be a broad spectrum chemoprotective agent and effective against cisplatin-induced nephrotoxicity and ototoxicity (Fouladi *et al*, 2008; Hartmann *et al*, 2000). Importantly, co-treatment of patients with amifostine and cisplatin does not decrease the efficacy of cisplatin (Korst *et al*, 1998). In male rats treated with cisplatin, amifostine co-treatment conferred partial protection against cisplatin-induced testicular damage and germ cell death (Lirdi *et al*, 2008). However, whilst amifostine has a partial protective effect on the seminiferous epithelium of male rats against doxorubicin exposure, long term fertility was not improved and there was increased early embryo loss suggesting that the quality of the sperm DNA was compromised (Vendramini *et al*, 2012; Vendramini *et al*, 2010). One study has examined amifostine in the ovary against cyclophosphamide treatment in female mice and found a decrease in apoptosis markers such as TUNEL in cotreated ovaries when compared to cyclophosphamide treatment alone. These mice were superovulated three weeks following treatment and reassuringly, the ovulation rates, fertilisation rates and embryo development up to the 4 cell stage were rescued in the co-treated group when compared to cyclophosphamide-only treatment (Barekati *et al*, 2012).

6.1.2.6. Antioxidants

The ability of antioxidants to act as chemoprotectants is currently garnering massive research attention. This is because chemotherapy agents such as doxorubicin and cyclophosphamide have shown to cause negative side effects such as cardiotoxicity, at least in part, through oxidative stress (Schimmel *et al*, 2004). For example, curcumin, a dietary supplement commonly known as tumeric, has been shown to reduce the negative side effects of cyclophosphamide on the lungs and kidney through its antioxidant and anti-inflammatory properties (Arafa, 2009; Venkatesan and Chandrakasan, 1995). Pretreatment with curcumin in males reduced sperm abnormalities following exposure to metronidazole (Singh *et al*, 2013). The ability of curcumin to protect the ovary against chemotherapy-induced damage is currently unclear.

Mesna (sodium 2-mercaptothanesulfonate) is an antioxidant which has been used to prevent haemorrhagic cysts, an adverse side effect often associated with cyclophosphamide treatment (Freedman and Ehrlich, 1984). It has been used in one study in rats to determine if it can prevent cisplatin-induced ovarian damage and it was determined that cisplatin reduces the number of AMH positive follicles and serum AMH levels, an effect which was prevented by mesna cotreatment, indicating that mesna may protect small growing follicles (Yeh *et al*, 2008). However, this is the only endpoint utilised by this study and so the ability of mesna to protect the ovary needs to be explored further.

Selenium and selenium-containing compounds are also being explored as potential chemoprotectants. Co-treatment with selenium has been found to have a protective effect against cisplatin damage in the kidney of rats through a decrease in oxidative stress (Ognijanovic *et al*, 2012). Selenium nanoparticles have recently been used to protect the testis against cisplatin in a rat *in vivo* model, with a significant improvement in sperm quality, spermatogenesis and serum testosterone levels with cotreatment (Rezvanfar *et al*, 2013). The effectiveness of selenium as a chemoprotectant in the ovary has not yet been established, although it has recently been shown to be effective against ischemia-induced damage in rat ovaries (Bozkurt *et al*, 2012).

6.1.2.5 Imatinib

Imatinib mesylate was the first receptor tyrosine kinase inhibitor to pass clinical trials. It is now the primary treatment for chronic myeloid leukaemia due to its inhibition of BCR-ABL (Fausel, 2007; Pytel *et al*, 2009) and is also effective against gastrointestinal stromal tumours (Eisenberg and Pipas, 2012). It selectively inhibits the tyrosine kinases Abl, c-kit and PDGF receptor (Steehgs *et al*, 2006). A study which used imatinib alongside cisplatin found a significant reduction in follicle death when compared to cisplatin alone (Gonfloni 2009). A later study published conflicting results, finding no protective effect of imatinib (Kerr *et al*, 2012). The difference in these two studies can potentially be explained by the differing drug concentrations used. The later study also found imatinib to be detrimental to follicle health alone although other recent evidence shows no significant damaging effect to either follicles or spermatagonia when imatinib was used at serum levels (Schultheis

et al, 2012). The ability of imatinib to protect the ovary against other chemotherapeutic agents including doxorubicin is currently unknown.

6.1.3 Aims

The aim of the work detailed in this chapter was to determine if imatinib can protect the ovary from cisplatin or doxorubicin induced damage.

6.2 Materials and Methods

6.2.1. Animals

C57Bl6J females were used for all experiments detailed here as described in Chapter 2.

6.2.2 Imatinib stock solution

Imatinib mesylate (Catalogue number CAYM13139, Cayman Chemicals through VWR International) was made up in sterile water (Sigma Aldrich, W1515) in a class II fume hood to the concentration of 3mg/ml. This was aliquoted out in 10µl quantities and frozen at -20°C. Stocks were replaced every 6 months.

6.2.3. Newborn ovary culture

Newborn ovaries were dissected and cultured for 6 days as described in Chapter 2 and 3. For the cultures containing imatinib mesylate, imatinib was added to produce a final concentration of 3µg/ml. This concentration was chosen due to preliminary dose test results (Sampurna Ghosh and Norah Spears, unpublished results). Cisplatin or doxorubicin exposure was limited to Day 2 of culture as in previous experiments, with imatinib present throughout Days 1-3 of culture to maximize any potential protective capacity of the drug. Cisplatin was added to produce a final concentration of 0.5µg/ml, while doxorubicin was added to produce a final concentration of 0.05µg/ml. These mid-range doses were chosen as both induced the appearance of a similar percentage of unhealthy follicles (around 30%), without causing the extensive damage to the ovary found after exposure to the highest doses. After the 3 days of imatinib exposure, ovaries were moved into drug free culture for a further 3 days

(Days 4-6) with 50% of medium changed every other day. Seven ovaries were analysed in each treatment group which were taken from 4 independent cultures.

6.2.4. P4 ovary culture

P4 (Postnatal day 4) mouse ovaries were collected and dissected in the same way as the newborn ovary culture method. They were cultured in the same drug concentrations and conditions as described above. Five ovaries were analysed in each treatment group taken from 3 independent cultures.

6.2.5. Histology

Ovaries were collected at the end of culture and fixed in 10% buffered formalin solution. They were processed, embedded and sectioned as described in chapter 2. For the newborn ovaries, every 6th section was stained with haematoxylin and eosin then analysed. For the P4 ovaries, every 12th section was stained and analysed. This was because the P4 ovaries are larger than the newborn ovaries and so fewer sections were sampled. Follicles were analysed as described in Chapter 2.

6.2.6. Statistical analysis.

All data were analysed using Graphpad Prism. One way ANOVA determined if significant differences were present across treatments, followed by Bonferroni post hoc tests where ANOVA was statistically significant. Post hoc tests determined the effect of treatment relative to its control.

6.3 Results

6.3.1. Newborn ovary cultures

Newborn ovaries were treated with:

control,

cisplatin,

doxorubicin,

imatinib,

imatinib and cisplatin or

imatinib and doxorubicin.

Representative pictures of sections are shown in Fig 6.1. Total number of follicles counted in each treatment group is shown in Table 6.1. Imatinib treatment alone led to a small reduction in the percentage of unhealthy follicles when compared to control, although this was not significant (Fig 6.2A). There was a significant reduction in the percentage of follicles classified as unhealthy when imatinib was added in conjunction with cisplatin when compared to cisplatin treatment alone (Fig 6.2A). Imatinib tended to lead to a reduction in the percentage of unhealthy follicles present during exposure to doxorubicin (9% reduction), but this was not significant. The total number of follicles present was significantly higher in imatinib treatment when compared to control (Fig 6.2B). The same trend occurred in the cisplatin- ($p=0.38$) and doxorubicin- ($p=0.54$) treated cultures but was not significant (Fig 6.2B).

The significant difference in follicle number found in the cultures of newborn ovaries could be due to either an increase in follicle survival and/or an effect on follicle

formation, as there is still some formation occurring in the first three days postnatal life (Pepling and Spradling, 2001). This also occurs in culture (Federica Lopes, unpublished results) and therefore during the window of imatinib exposure. The experiment was repeated by culturing 4 day old ovaries (P4) as follicle formation should have finished by this point. This would mean that any effect of imatinib found would have to be due to an effect on follicle survival.

6.3.2. P4 cultures

P4 ovaries were cultured as described above, representative pictures of which are shown in Fig 6.3. Total number of follicles counted in each treatment group is shown in Table 6.2. Imatinib treatment alone had no significant change on the percentage of follicles classified as unhealthy when compared to control (Fig. 6.4A). There was a reduction in the percentage of follicles classified as unhealthy when imatinib was added in conjunction with cisplatin when compared to cisplatin treatment alone, however this was not significant. There was no significant difference in the number of follicles present in the imatinib-treated group compared to the control, indicating that the difference in follicle number seen in the newborn ovary cultures is likely due to an effect on follicle formation (Fig 6.4B).

Follicle distribution was also examined in both the newborn and P4 ovaries to see if there was any change (Fig 6.5). This is because tyrosine kinase signalling has been heavily implicated in primordial follicle activation (Nilsson *et al*, 2006; Nilsson and Skinner, 2004). For clarity, the doxorubicin and the doxorubicin and imatinib cotreatment groups are not shown (similar but not significant trends seen as shown in

Fig 6.5). Imatinib treatment alone caused a significant decrease in the percentage of follicles in newborn ovaries classified as primary compared to control. Imatinib cotreatment with cisplatin caused a similar significant decrease in the percentage of follicles in newborn ovaries classified as primary compared to cisplatin treatment alone (Fig 6.5A). There was a corresponding but non-significant increase in the percentage of primordial follicles (PMF) in the imatinib group compared to control as well as in the imatinib cotreatment group when compared to cisplatin alone. There were no significant differences in the follicle distribution when examined in the P4 ovaries (Fig 6.5B).

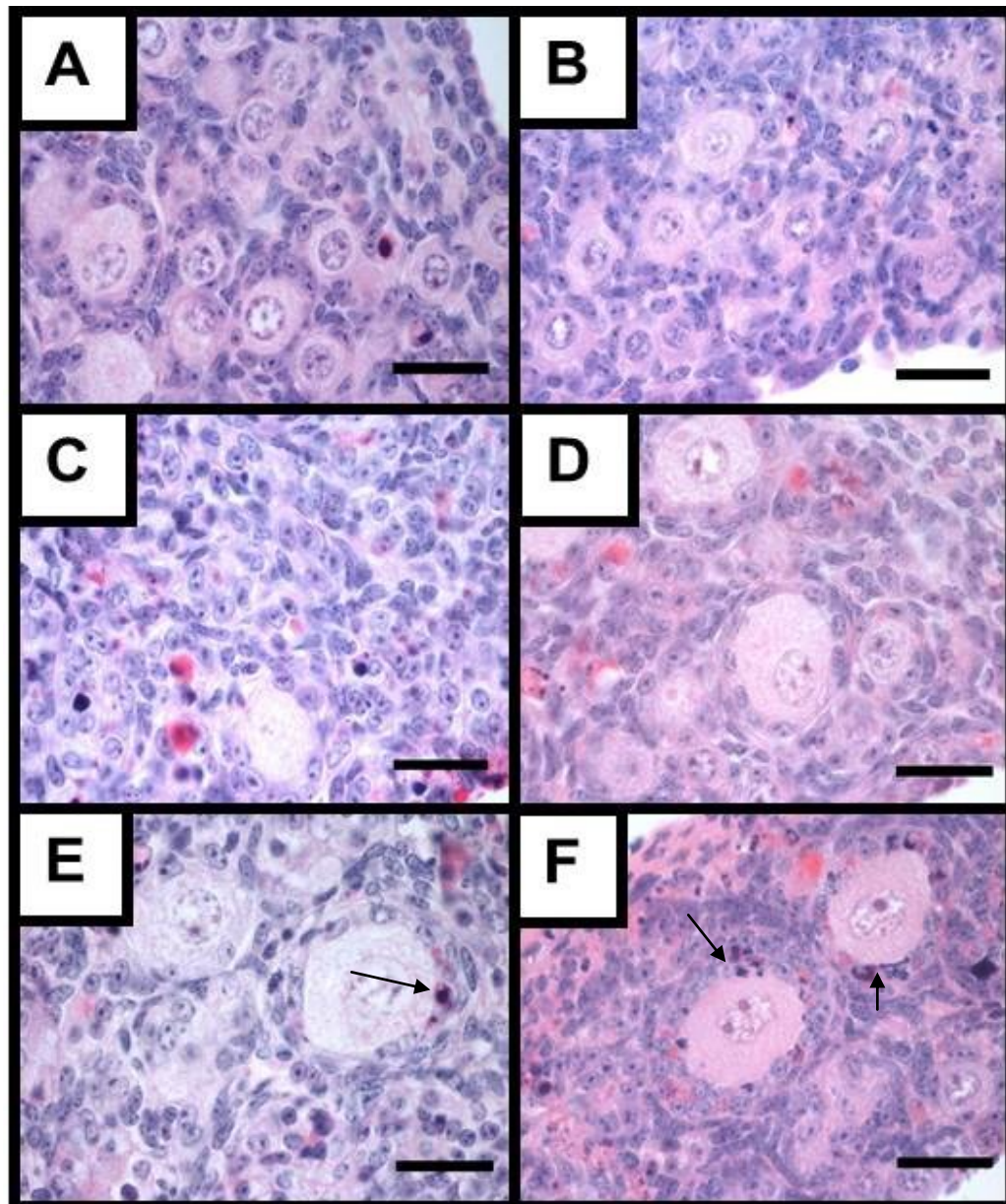


Figure 6.1. Representative photomicrographs of haematoxylin and eosin stained sections from newborn mouse ovaries treated with **(A)** control, **(B)** 3µg/ml imatinib, **(C)** 0.5µg/ml cisplatin, **(D)** cisplatin and imatinib co-treatment, **(E)** 0.05µg/ml doxorubicin and **(F)** doxorubicin and imatinib co-treatment. Scale bars represent 25µm. Black arrows indicate pyknotic granulosa cells.

	Total number of follicles counted	Total number counted classified as healthy	Total number counted classified as unhealthy (%)
Control	633	559	74 (11)
3µg/ml imatinib	1525	1425	100 (7)
0.5µg/ml cisplatin	891	639	252 (28)
Cisplatin and imatinib co-treatment	1237	1153	84 (7)
0.05µg/ml doxorubicin	703	535	168 (24)
Doxorubicin and imatinib co-treatment	1032	864	168 (16)

Table 6.1. Total number of follicles counted in newborn mouse ovaries cultured in the presence of absence of imatinib (n=7 ovaries in each treatment group) as well as the total number of follicles classified as healthy or unhealthy.

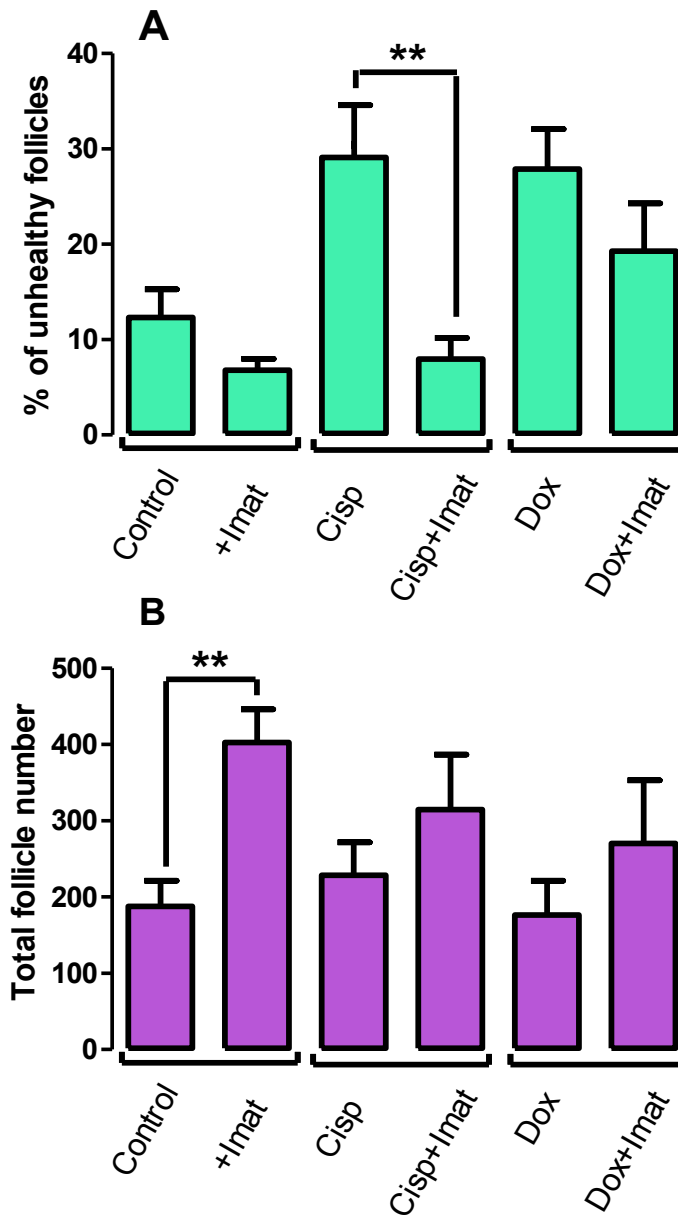


Fig 6.2. Control, cisplatin-treated (0.5 μ g/ml) and doxorubicin-treated (0.05 μ g/ml) cultured newborn mouse ovaries were cultured for six days in the presence or absence of imatinib. **(A):** Percentage of unhealthy follicles; **(B):** Total number of follicles. Bars denote mean \pm sem; n=7 for all groups, stars denote significant differences relative to control (**p<0.01).

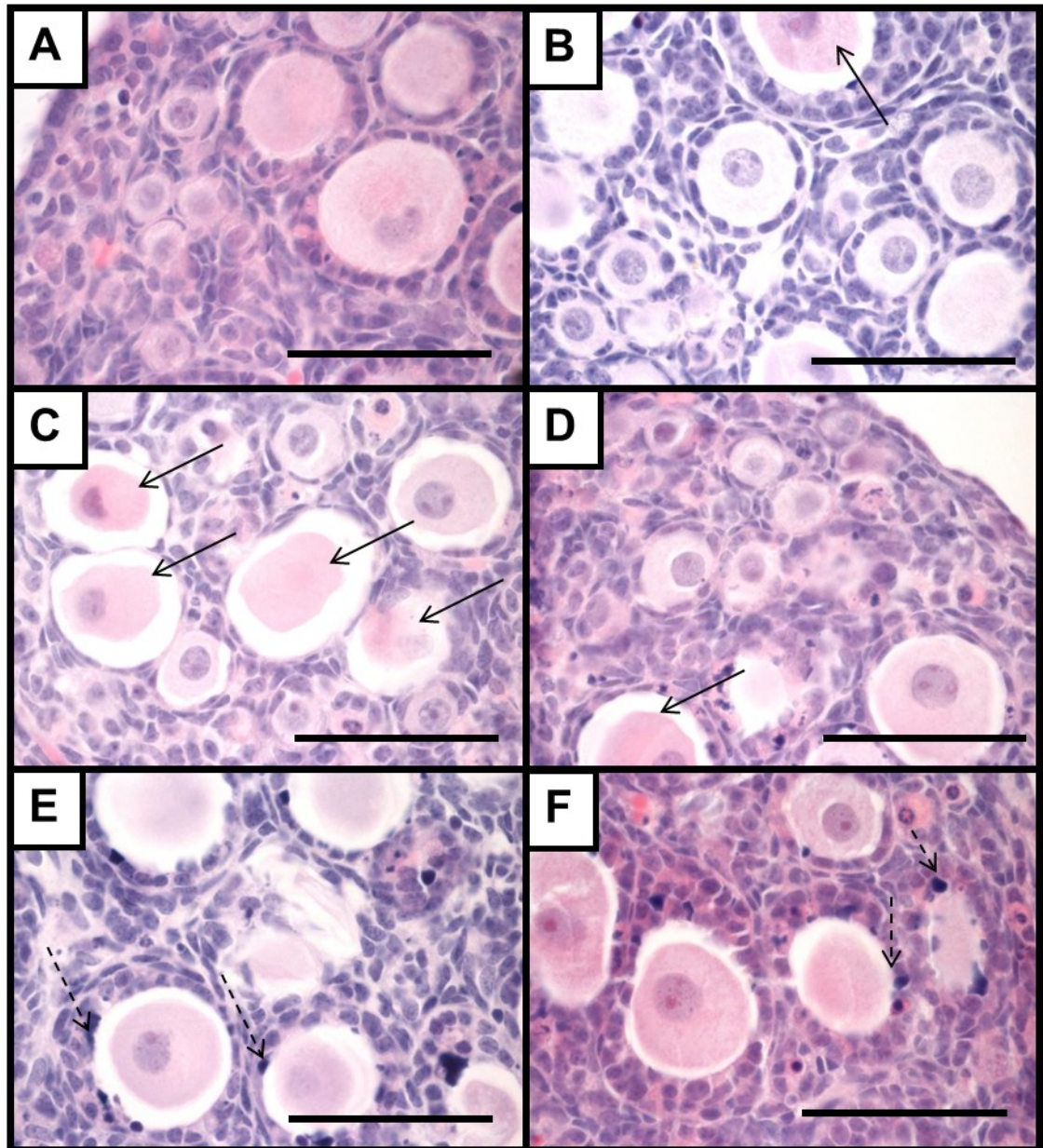


Fig 6.3. Photomicrographs of haemotoxylin and eosin stained sections from P4 mouse ovaries treated with (A) control, (B) 3 μ g/ml imatinib, (C) 0.5 μ g/ml cisplatin, (D) cisplatin and imatinib co-treatment, (E) 0.05 μ g/ml doxorubicin and (F) doxorubicin and imatinib co-treatment. Scale bars represent 50 μ m. Solid black line arrows indicate unhealthy oocytes. Dashed black line arrows indicate follicles with pyknotic granulosa cells.

	Total number of follicles counted	Total number of follicles counted classified as healthy	Total number of follicles counted classified as unhealthy (%)
Control	1095	1003	92 (8)
3µg/ml imatinib	721	669	52 (7)
0.5µg/ml cisplatin	556	335	221 (40)
cisplatin and imatinib co- treatment	415	319	96 (23)
0.05µg/ml doxorubicin	545	390	155 (28)
doxorubicin and imatinib co- treatment	259	160	99 (38)

Table 6.2. Total number of follicles counted in P4 mouse ovaries cultured in the presence of absence of imatinib (n=5 ovaries in each treatment group) as well as the total number of follicles classified as healthy or unhealthy.

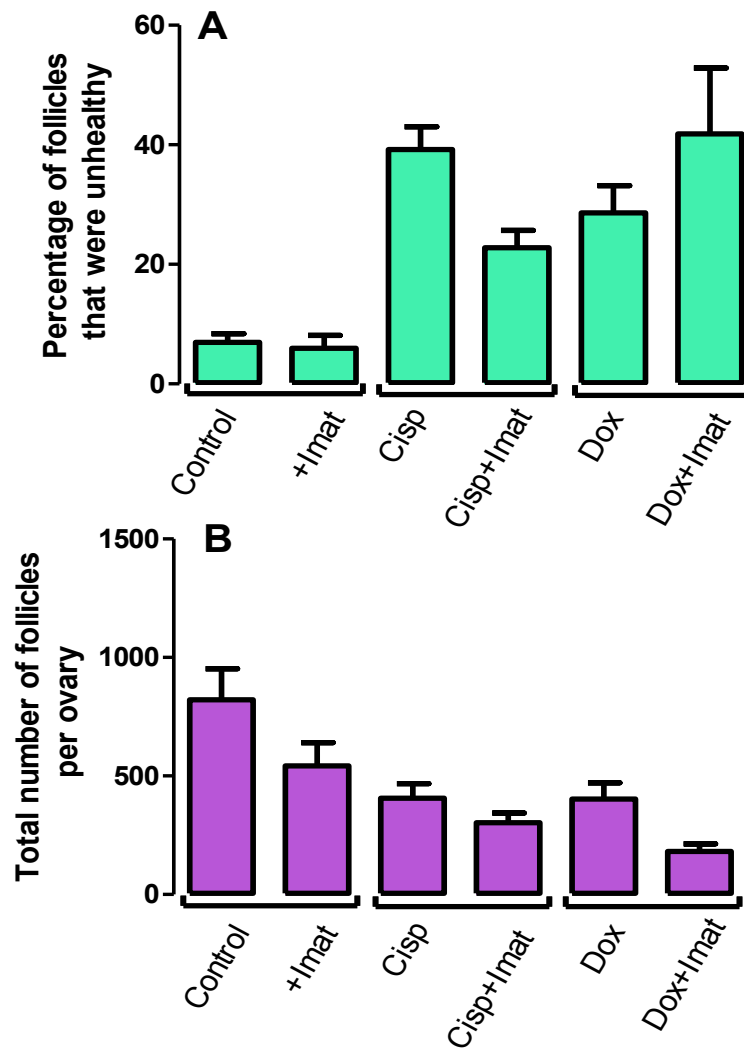


Fig 6.4 Control, cisplatin-treated (0.5 μ g/ml) and doxorubicin-treated (0.05 μ g/ml) P4 mouse ovaries were cultured for six days in the presence or absence of imatinib. **(A):** Percentage of unhealthy follicles; **(B):** Total number of follicles. Bars denote mean \pm sem; n=5 for all groups.

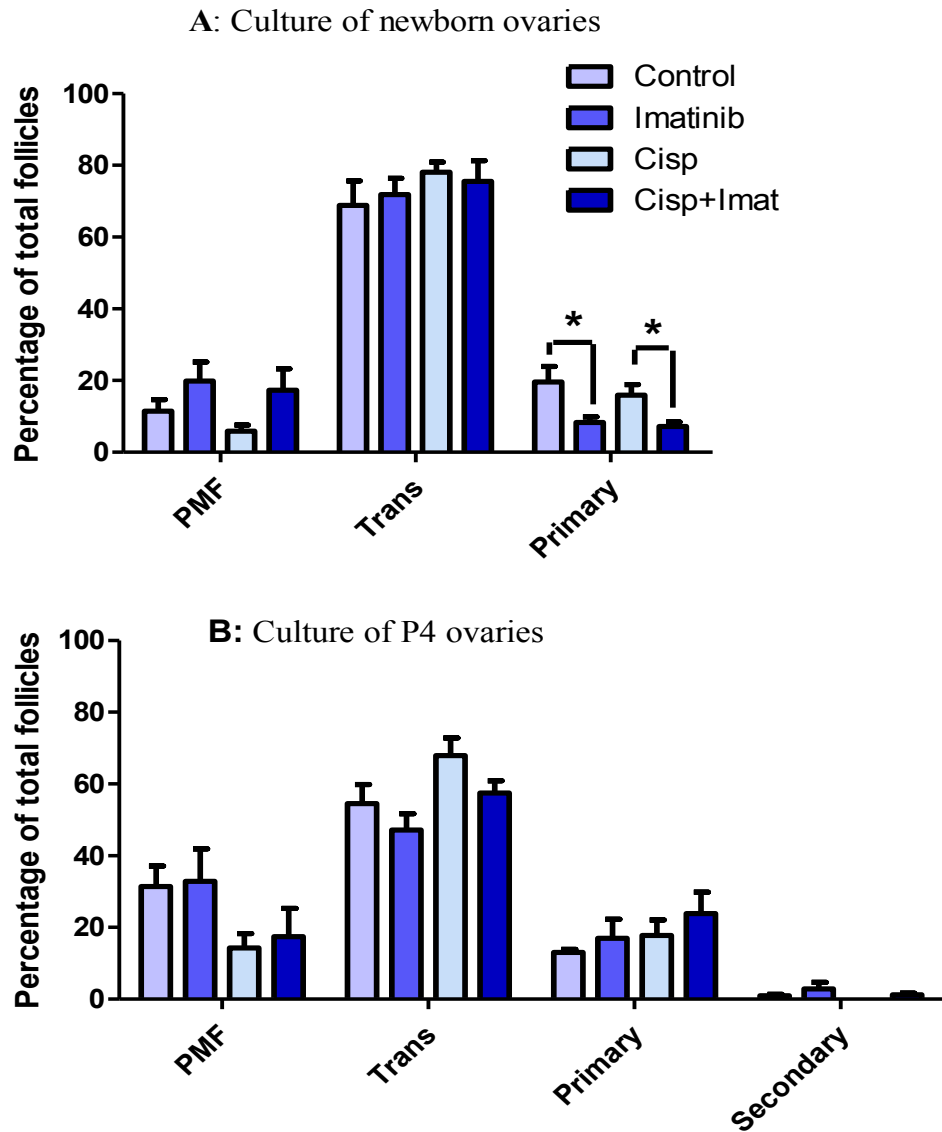


Fig 6.5. Follicle distribution in (A) newborn and (B) P4 murine ovaries comparing control medium to imatinib treatment and cisplatin treatment to cisplatin and imatinib cotreatment over six days of culture. Bars denote mean \pm sem, n=5, stars denote significant differences (*p<0.05).

6.4 Discussion

Imatinib mesylate was used in conjunction with cisplatin and doxorubicin, to determine if imatinib could protect the ovary from chemotherapy-induced follicle damage. Results here indicate that imatinib is effective against cisplatin- but not doxorubicin-induced follicle loss. Imatinib also increases follicle number, which is likely due to an effect on follicle formation.

6.4.1. Imatinib's protective effect

Recent work has suggested that the imatinib can reduce the toxic effect of cisplatin on the ovary through its inhibition of c-Abl (Gonfloni *et al*, 2009). Results here provide evidence of a reduction by imatinib of the adverse effect of cisplatin on follicle health. This protection is specific, with no significant protection found against doxorubicin-induced damage. The effect is clearer in the newborn ovaries than in the P4 ovaries, potentially due to a larger sample size in the newborn experiments. This is in contrast to (Kerr *et al*, 2012), who did not find a significant protective effect of imatinib against cisplatin-induced damage, possibly due to the difference in drug dosages between the two studies (10 μ M imatinib, 20 μ M cisplatin in (Kerr *et al*, 2012); 5.09 μ M imatinib and 1.67 μ M cisplatin here). The fact that the adverse effects of cisplatin were inhibited by imatinib but not those of doxorubicin is further evidence that these drugs act on the ovary via different mechanisms.

Imatinib is thought to provide ovarian protection against cisplatin damage by inhibiting c-Abl, a tyrosine kinase which promotes accumulation of p63, which in turn activates cell death following high levels of DNA damage (Gonfloni, 2010;

Gonfloni *et al*, 2009). c-Abl is ubiquitously expressed in both the nucleus and cytoplasm of cells and has recently been implicated to be an important component in the response to DNA damage (Shaul and Ben-Yehoyada, 2005). Inhibition of c-Abl prevents a subsequent accumulation of TAp63, high levels of which is essential for cisplatin-induced oocyte death (Kim *et al*, 2013).

In addition, imatinib treatment alone did not have a deleterious effect on the ovary, in agreement with some recent work (Maiani *et al*, 2012; Schultheis *et al*, 2012) although in contrast to another study (Kerr *et al*, 2012). Kerr *et al* (2012) used a higher concentration of imatinib, which may explain the discrepancy (10 μ M compared to 1 μ M in Maiani *et al* (2012) and approximately 5 μ M here). The concentration used here is the same as that used by another very recent study (Kim *et al*, 2013), which also found this to be the most effective and non-damaging concentration to use.

6.4.2. Imatinib and follicle formation

Imatinib treatment alone in newborn ovaries caused a significant increase in the number of follicles present. There were two potential explanations for this. The first is that imatinib is a pro-survival factor for follicles in itself. The second is that it has a stimulatory effect on follicle formation. Follicle formation still continues *in vivo* through to postnatal day 3, which spans the drug exposure window (Pepling and Spradling, 2001; Smitz and Cortvrindt, 2002). Culture of P4 ovaries showed no significant increase in the number of follicles present indicating that it is likely that

imatinib caused the increase in follicle number in newborn ovaries through an effect on formation.

Imatinib inhibits tyrosine kinases including Abl, c-kit and platelet derived growth factor receptor (PDGF-R) and so it is likely that the effect on follicle formation is mediated through one of these factors. Relatively little is known about the action of tyrosine kinase's on follicle formation (Pepling, 2006). Addition of SCF (Stem Cell Factor also known as Kit ligand) to hamster ovaries accelerates follicle formation and conversely inhibition of SCF decreases follicle formation (Wang and Roy, 2004). As imatinib inhibits c-kit which is the receptor for SCF, the opposite effect to that seen here would be expected. A study which injected an antibody that selectively inhibited c-kit in mouse ovaries, found no evidence of an effect of c-kit on follicle formation (Yoshida *et al*, 1997). One way of establishing which tyrosine kinase is responsible for this effect on follicle formation would be to culture newborn ovaries with imatinib and each of these factors to see if the effect is reversed.

6.4.3 Imatinib and follicle recruitment

One of the major events that tyrosine kinases have been implicated in within the ovary is follicle activation. Kit ligand and PDGF have both shown to upregulate the transition from primordial follicle to primary (Nilsson *et al*, 2006; Nilsson and Skinner, 2004) and inhibition of c-kit has been demonstrated to prevent primordial follicle activation (Yoshida *et al*, 1997). There was a significant decrease in the percentage of primary follicles in cisplatin- and imatinib- treated newborn ovaries when compared to cisplatin- treatment alone. There is also a non- significant increase

in the number of primordial follicles in the cisplatin- and imatinib-treated group compared to cisplatin alone which could suggest a decrease in primordial follicle recruitment. In contrast, this effect is not seen in P4 ovaries. This could be due to the reduced n number which was used in the P4 experiment (carried out at the end of the PhD). Another potential explanation is that, although inhibition of c-kit has been demonstrated to be important for preventing primordial follicle development, the timing of inhibition is crucial, with a significant effect only seen within the first 5 days post-partum (Yoshida *et al*, 1997).

As cyclophosphamide has recently been demonstrated to cause increased recruitment of primordial follicles, and AS101 co-treatment leading to a concurrent decrease (Kalich-Philosph *et al*, 2013), whether imatinib could have an impact on follicle activation is worthy of consideration. Aside from examining the follicle distribution, there are other methods to evaluate this. Using immunohistochemical markers for members of the PI3K/PTEN/Akt pathway for example, which have recently been demonstrated as important for follicle activation (Jagarlamudi *et al*, 2009).

6.4.4. Imatinib as a clinical treatment

Imatinib is currently used to treat chronic myeloid leukemia as well as gastrointestinal stromal tumours (Fausel, 2007; Patel, 2013). Its use in conjunction with cisplatin has not been tested clinically and there is concern that it could affect the anti-tumour activity of cisplatin. This needs to be tested as this would limit imatinib's clinical use as a protective agent.

Another concern is that whilst imatinib may protect oocytes from cisplatin-induced loss, the oocytes may still be damaged. Instead of preventing cisplatin-induced DNA damage, imatinib may be preventing the ovary from clearing oocytes which are damaged by inhibiting the activation of cell death pathways. A recent study has shown that cisplatin-induced DNA damage in oocytes remains present following imatinib co-treatment (Kim *et al*, 2013). Whether or not oocytes have the ability to repair this kind of DNA damage remains to be seen but failure to do so could have downstream effects on fertilisation and embryo viability.

6.4.5. Conclusion

Imatinib mesylate confers specific ovarian protection against cisplatin- but not doxorubicin- induced early follicle damage. Imatinib also appears to have an effect on promoting follicle formation.

CHAPTER 7.

DISCUSSION

7.1. Summary of Results

Chemotherapeutic agents lead to follicle loss and premature ovarian failure in premenopausal women; this thesis set out to determine specific mechanisms through which this occurs. Cisplatin is an alkylating-like chemotherapeutic agent which is considered to be moderately ovotoxic and is commonly used in premenopausal women for the treatment of ovarian, bladder and cervical cancer. Initial experiments compared the action of cisplatin in two different model species, the mouse and the cow. Both models demonstrated an increase in the percentage of unhealthy follicles following treatment and these follicles were classified as unhealthy due to poor oocyte morphology. Similar results were found in both species and due to the high number of primordial follicles in the mouse ovary, it was decided that subsequent experiments would be conducted using the mouse.

The major aim of this project was to establish the ovarian cell type and follicle classes affected by chemotherapy agents. To do this, neonatal mouse ovaries were cultured *in vitro*, exposed to cisplatin and examined histologically. It was determined that cisplatin caused an increase in the number of follicles classified as unhealthy and total follicle loss. Follicles which were classified as unhealthy primarily had morphologically unhealthy oocytes, suggesting that cisplatin acts directly on the germ cell (Fig 7.1). This effect was initially seen in the primary follicles, indicating that the growing follicles may be more vulnerable to cisplatin-induced toxicity. The action of cisplatin was compared within the same experimental system to another chemotherapeutic drug, doxorubicin, which is also commonly used in premenopausal women for the treatment of breast cancer and Hodgkins lymphoma. The effect of doxorubicin was different, with follicles classified as unhealthy primarily due to the

presence of pyknotic granulosa cells. The follicle class affected was also more general than cisplatin (Fig 7.1). The difference in these two drugs indicates that these two drugs are likely to be affecting the ovary through different mechanisms.

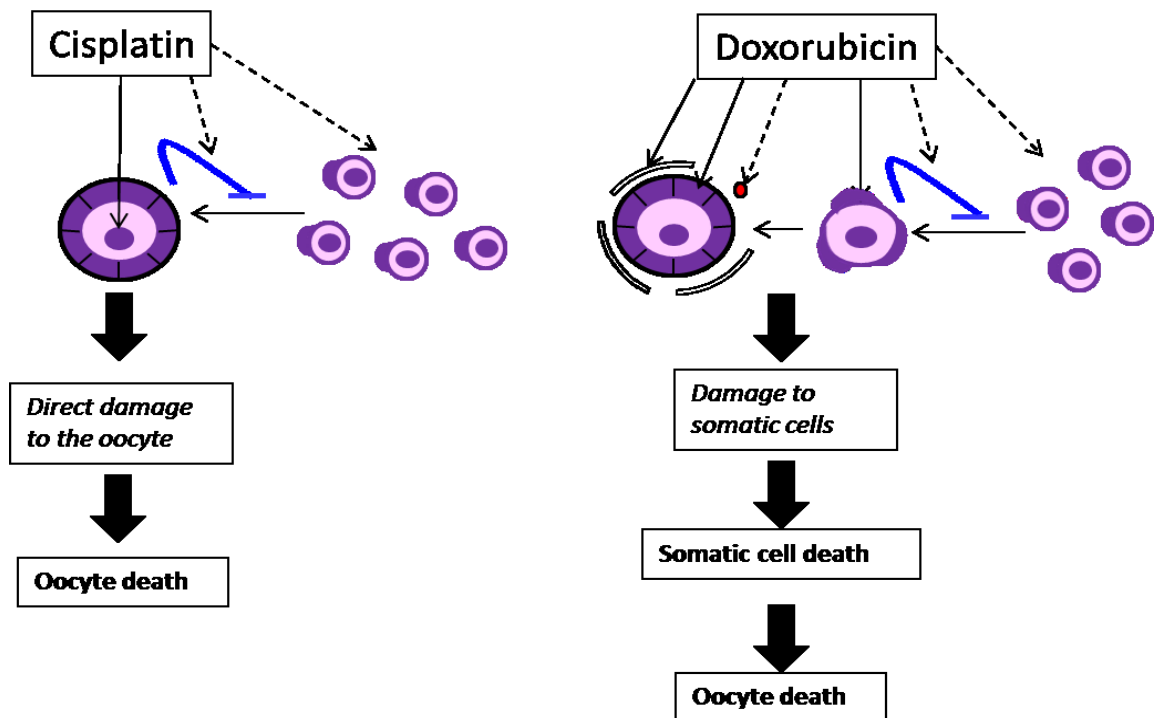


Fig 7.1. Summary of the different mechanisms through which cisplatin and doxorubicin cause follicle loss. Cisplatin acts primarily on the oocyte of the growing primary follicles, leading to oocyte death and follicle loss. The follicle class affected is more general following doxorubicin exposure, with the site of action primarily the granulosa cells. The stromal cells and possibly the blood vessels are also damaged following doxorubicin treatment. It is unclear whether either drug directly causes primordial follicle loss or if there is an increase in primordial follicle activation after exposure (as indicated by the dashed lines).

The molecular mechanism through which these two drugs act was then investigated. Results indicate that the primary action of both of the drugs is apoptosis, with both causing an increase in the protein expression of cleaved PARP, a marker of apoptosis. The expression of cleaved PARP was examined by immunohistochemistry and indicated that doxorubicin causes apoptosis in the somatic cells, including the granulosa cells and the stroma. Further examination with cleaved caspase-3, another marker of apoptosis, demonstrated increased granulosa cell apoptosis following doxorubicin exposure. These results fit well with the histological data and altogether demonstrate that doxorubicin causes follicle damage through apoptosis of the somatic cells. The molecular results for cisplatin were less clear, although cleaved PARP protein levels significantly increase indicating cisplatin also causes apoptosis. A potential apoptosis pathway through which cisplatin and doxorubicin could act is described in Fig 7.2.

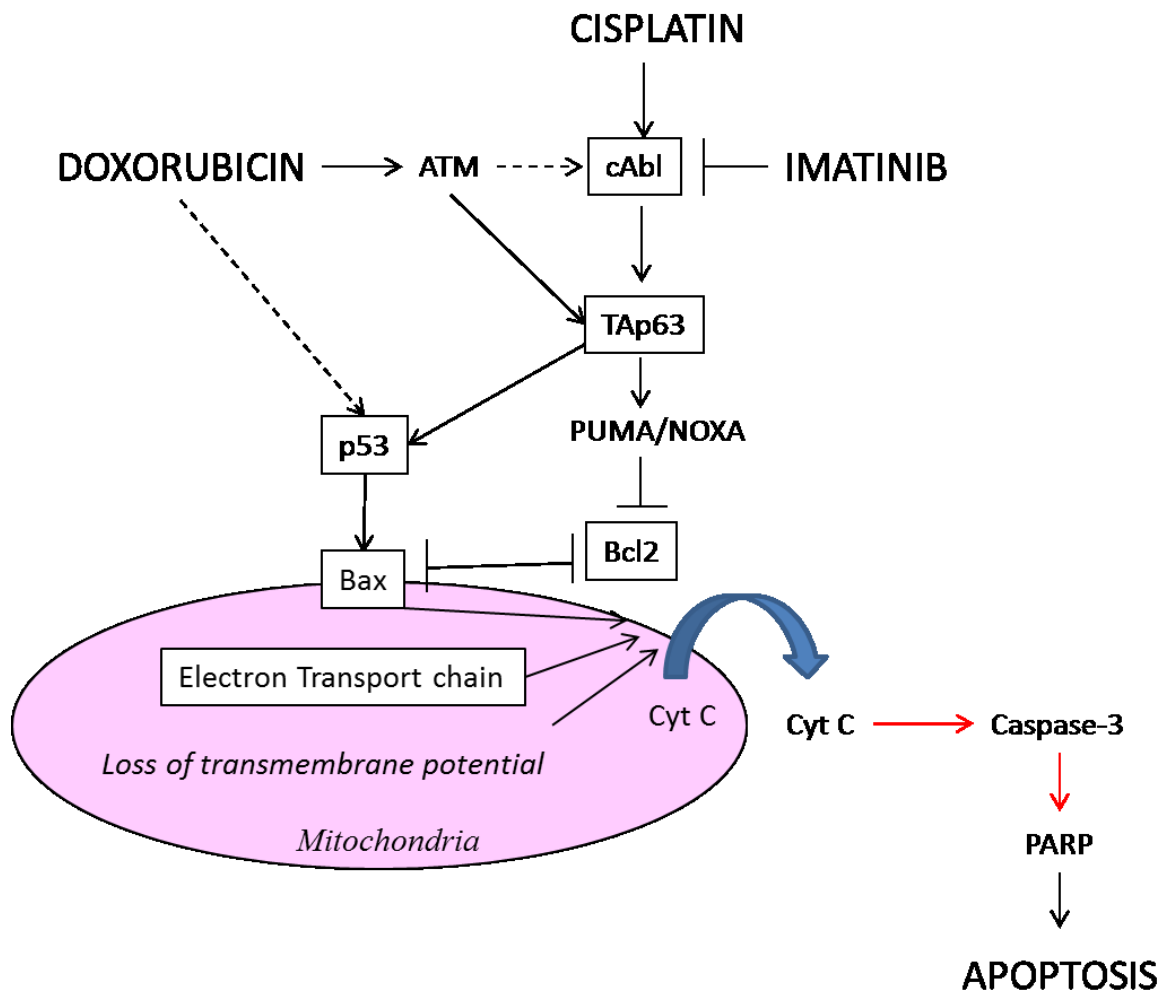


Fig. 7.2. Potential mechanism by which cisplatin and doxorubicin could cause cell death. Cisplatin's initiation of apoptosis is dependent on cAbl, which is inhibited by imatinib. Doxorubicin may act through ATM, which provides an alternate route of activating TAp63, thereby explaining why imatinib co-treatment fails to rescue doxorubicin-induced damage. Through TAp63 activation (and potentially p53), both drugs cause apoptotic cell death. Dashed lines indicate potential activations, red arrows indicate cleavage activation.

Protecting the ovary directly from damage induced by chemotherapeutic agents would be the ideal solution for preventing premature ovarian failure. Imatinib

mesylate is a chemotherapeutic agent which has been used in other laboratories as a protective treatment against cisplatin (Gonfloni *et al*, 2009; Kim *et al*, 2013), although its effectiveness is controversial. Results here demonstrate that imatinib has a protective effect against cisplatin treatment only, with no protection conferred against doxorubicin-induced toxicity (potential mechanism outlined in Fig 7.2). An interesting side effect of imatinib treatment was an increase in the total number of follicles present in the ovary. Further experiments culturing postnatal day-4 ovaries with imatinib removed this difference, indicating that this difference in follicle numbers in the newborn ovary cultures is almost certainly due to an effect on follicle formation. Imatinib is a tyrosine kinase inhibitor, and so this result highlights an importance for tyrosine kinase signalling in follicle formation, with, relatively little known about this at present.

The results of this thesis overall demonstrate that two chemotherapeutic agents commonly used in premenopausal women, cisplatin and doxorubicin, have different effects on the mouse ovary. Cisplatin causes oocyte-specific damage, whilst doxorubicin in contrast causes apoptosis of the granulosa cells as well as increased stromal death. Furthermore, the putative protective agent imatinib has a protective effect against cisplatin but not doxorubicin. These results suggest that different chemotherapeutic agents will act through a variety of mechanisms on the ovary and so any protective treatment may have to be designed in a patient specific manner.

7.2. Future Work and Considerations

7.2.1. Extending the mouse *in vitro* model

The mouse ovary has proven to be an invaluable tool for research into human folliculogenesis. In terms of *in vitro* culture, the mouse is the best defined ovarian model available with live offspring produced from cultured primordial follicles (O'Brien *et al*, 2003). Other advantages of using the mouse as a model include high material availability and the ease at which the mouse can be genetically manipulated, allowing for the role of specific genes to be determined. The material available is also extremely similar as it is from mice which are the same age, from the same inbred line and raised in the same environmental conditions. However, when using the mouse as a model, it is important to consider species differences and limitations. The human ovary is larger, with much more stromal tissue than the mouse. In contrast to the monovular humans, the mouse is polyovular, with a short oestrous cycle and a short period of folliculogenesis. There are some issues with using human ovarian tissue experimentally, the first of which is limited tissue availability. Also, whilst there have been great advances in the culture of human ovarian tissue and follicles, this is still not a well-defined experimental model (Telfer and Zelinski, 2013). Overall, I think that the work described here has produced a good model for the initial investigation of chemotherapy drug toxicology but that it has limitations. Therefore, some of this work should be repeated using human ovarian tissue, to ensure the removal of species differences in their response to chemotherapeutic agents. As tissue availability is such an issue, using another model species which is more closely related to humans than mice, is also an option for further extending this work. One possibility would be to use a non-human primate model. The non-human

primate ovary is the closest model to humans, as it has a protracted period of folliculogenesis *in vivo* as well as a long follicular stage in the menstrual cycle (Gougeon, 1996; Telfer and Zelinski, 2013).

The work presented in this thesis was also solely *in vitro*. Whilst this is a valuable tool, as it allows for more specific evaluation of direct drug actions on the ovary, there are other factors to consider in patients. The amount of drug which an ovary is exposed to *in vivo* is difficult to extrapolate solely from serum dosages, as the blood supply to the ovary is more limited, and less well defined, than that of other organs. Interactions with the hypothalamic-pituitary axis are also a consideration as any detrimental effect of chemotherapy on the brain could have a downstream negative effect on the ovary. However, whilst hypopituitarism (leading to a decrease in gonadotrophin secretion) has been reported following cranial irradiation, it is not currently linked to chemotherapy treatment (Darzy and Shalet, 2005). The *in vitro* environment does mimic the *in vivo* one to an extent, as healthy offspring can be produced in the mouse following oocyte culture. However, it is only to an extent, and there are many factors and interactions in the ovary which are still unclear. The advantage of using the *in vitro* neonatal culture system used in this thesis is that the follicle population present is fairly uniform and so more specific effects of drug treatment can be established. It is difficult to expose these ovaries *in vivo* due to the general toxicity of chemotherapy agents on neonatal mice and so *in vivo* experiments would have to utilise adult females. Using adult females as an *in vivo* model is useful for determine the effect of chemotherapy on long term fertility but less so for determining specific mechanisms of drug action due to the heterogeneity of the follicle population in these mice. Therefore, a good extension to the work in this

thesis, which focused on more specific drug actions, would be to confirm the long term effect on fertility of chemotherapeutic agents *in vivo* using adult mice.

Another factor which this model has not yet taken into account is drug combinations. It is rare that a patient is ever exposed to a single drug treatment, instead most agents are used as combination therapy. Utilising commonly used combinations for premenopausal women such as CMF (cyclophosphamide, methotrexate and 5-fluorouracil) is another logical next step, as this will be more reflective of the clinical conditions. As cisplatin and doxorubicin here targeted different cell types in the ovary, their use in combination could potentially have more than additive effects. This is now being explored by others in the laboratory.

7.2.2 Protective treatments

The use of protective agents which could directly shield the ovary from the damaging effects of chemotherapy agents is gathering increasing research attention. Imatinib is perhaps now the most studied and appears, at least in some studies including this one, to be effective against cisplatin administration (Gonfloni *et al*, 2009; Kim *et al* 2013). With any putative protective treatment there are a number of clinical concerns which will need to be addressed. A major concern with imatinib, and potentially some of the other putative treatments, is that rather than preventing the damage that chemotherapy agents are inducing in oocytes, it is instead preventing them from dying from this damage. This would mean that, unless the oocyte is able to repair this damage itself, any detrimental effects would persist and would potentially be conferred to the embryo and hence the offspring. This is obviously far from ideal and so another approach would be to try and prevent the drug causing damage in the first

place. Such approaches could involve preventing cellular uptake (presumably difficult as cisplatin and doxorubicin appear to enter the cell through passive diffusion) or preventing the drug from entering the nucleus and causing DNA damage.

There are of course concerns that the protective agent would negatively interact with the anti-cancer ability of the chemotherapy drugs, something which would absolutely need to be established before their clinical use. Targeting the protective agent to the ovary would be one way of hopefully minimising this eventuality. So far the majority of studies investigating protective agents have done so either *in vitro* or, if *in vivo*, using injection/IV and thus serum exposure. One approach to more directly target the ovary would be to use an implant located adjacent to the ovary, an approach which has been successfully tried in one study in primates (Zelinski *et al*, 2011). Nanoparticles are a method which is being investigated to target chemotherapy to tumour sites and could potentially therefore be utilised to localise a protective agent to the ovary. One way to use these is to load magnetic nanoparticles with a drug, which can be localised to a specific area of the body on application of an external magnetic field (Deok Kong *et al*, 2012; Lee *et al*, 2013).

7.2.3. Mutagenesis, epigenetics and transgenerational changes

Cisplatin and doxorubicin have both been shown to cause an increase in the number of unhealthy follicles and follicle loss (Chapter 3). However, at doses equivalent to those seen in patients, there are still follicles remaining following exposure to either drug which look morphologically healthy. It is important to consider if the oocytes of primordial follicles which remain after drug treatment are healthy and capable of

correct embryonic development, as these are the oocytes which represent the germ cell reserve in patients. It is generally unlikely for patients to opt to become pregnant during or immediately after treatment, and therefore it is this follicle reserve which is the most likely to produce offspring.

Chemotherapeutic agents are mutagenic to somatic cells, causing abnormalities including chromosomal breaks, gene mutations and aneuploidy (Degrassi *et al*, 2004). This is highlighted by the incidence of secondary malignancies in patients following treatment (Borgmann *et al*, 2008). There is concern that these agents could also have similar effects on the germ cells which could influence fertilisation, viability of pregnancies and problems with the offspring including malformations. There is also the possibility that these agents compromise the cytoplasmic competence of the oocyte as well as the DNA. Most of the information on germ cell mutagenesis is derived from animal studies, the majority of which have been conducted using males rather than females. This is because when a female is treated with a chemical agent, any embryonic lethality could be due to problems induced on the uterus or other aspects of their reproductive physiology, rather than specifically mutagenesis on oocytes. The majority of studies in humans reassuringly show no increase in foetal abnormalities in the offspring of parents who underwent chemotherapy treatment (Sanders *et al*, 1996; Signorello *et al*, 2012; Winther *et al*, 2012), although chromosomal damage and aneuploidy has been detected in the sperm of male survivors (Brandriff *et al*, 1994; Genesca *et al*, 1990). DNA-damaged spermatozoa can however still fertilize oocytes and achieve full-term pregnancies (Ahmadi and Ng, 1999; Gandini *et al*, 2004). In general, the main limitation of the

human studies is that there is little information about the type or dosage of treatment which the patient received (Winther and Olsen, 2012). Current studies also concentrate on major abnormalities, meaning that more subtle changes which could be occurring, such as altered methylation patterns or changes in transcription, are not being detected.

Cyclophosphamide and other alkylating agents are the most researched drug class in terms of their effect on fertility. An increase in abortions and foetal malformations was found in female mice which were treated with cyclophosphamide and subsequently mated, with the malformation rate 10 fold higher in treated groups when compared to the control (Meirow *et al*, 2001). A third of conceptions which were from oocytes exposed at the earliest stages of growth were malformed (Meirow *et al*, 2001). In males, cyclophosphamide alters the sperms chromatin structure as well as the sperm head protein composition (Codrington *et al*, 2007). Mating of cyclophosphamide-treated male rats to females leads to an increase in pre- and post-implantation embryo loss and an increase in malformed and growth retarded fetuses (Trasler *et al*, 1986).

7.2.3.1 Cisplatin and doxorubicin as mutagens

There is surprisingly little information on the capacity of cisplatin or doxorubicin to act as germ cell mutagens. Cisplatin causes dominant lethal mutations in female mice, resulting in increased early embryonic mortality (Witt and Bishop, 1996). Another study examining the effect of cisplatin on mature MII oocytes *in vivo* found treatment lead to a marked increase in aneuploidy (Higson *et al*, 1992). In male mice,

cisplatin induces chromosomal aberrations in spermatocytes (Choudhury *et al*, 2000). Male rats treated with subclinical doses of cisplatin and subsequently mated had an increase in pre- and post-implantation embryo losses and a significant number of malformed or growth retarded foetuses (Seethalakshmi *et al*, 1992). Doxorubicin has been identified as a female specific inducer of dominant lethal mutations, particularly in preovulatory oocytes in female mice (Arnon *et al*, 2001). A small but significant increase in chromosome translocations was detected in spermatogonial stem cells in mice following doxorubicin exposure (Meistrich *et al*, 1990).

7.2.3.2 Protective treatments and mutagenesis

The ability of imatinib to protect the follicle population against cisplatin induced damage has been previously discussed (Chapter 6). Whilst there is evidence here and in two other studies that imatinib may help alleviate cisplatin-induced toxicity, there is no evidence presently on whether the germ cells which appear to have been protected are actually healthy (Gonfloni *et al*, 2009; Kim *et al*, 2013). Exploring the developmental competence of oocytes which have been protected by agents such as imatinib will be a useful tool, to determine if these treatments are truly conferring a protective effect.

7.2.3.3. Epigenetic modifications

The ability of chemotherapeutic agents and other drugs to create epigenetic modifications is not well described, especially in the germ line. It is logical to expect that, as chemotherapy drugs cause DNA damage and alterations in DNA structure, this could have an impact on epigenetic modifications and gene imprinting.

Curcumin, a dietary agent which is a putative protective agent against cyclophosphamide-induced POF, is an inhibitor of DNA methyltransferase leading to DNA hypomethylation (Teiten *et al*, 2013). Sorafenib, another tyrosine kinase inhibitor primarily used for kidney cancers, also produces anti-tumour effects through epigenetic changes including histone modifications (Zhang *et al*, 2013). In terms of germ cells, cyclophosphamide exposure in males which were subsequently mated leads to disturbed methylation and changes in chromatin structure in the zygote (Barton *et al*, 2005). If changes such as these are not repaired, this could result in aberrant embryogenesis. The ability of chemotherapeutic agents to modify female germ cell epigenetics is currently unclear and therefore a huge potential research area.

7.2.3.4. Transgenerational effects

Transgenerational effects are also a concern, whether they are attributable to DNA damage, cytoplasmic changes or epigenetic modifications. Cyclophosphamide treatment in male rats leads to cerebral changes and behavioural deficits in the first and second generation of progeny as well as increased postnatal mortality and learning deficits in the third generation (Auroux *et al*, 1990; Dulioust *et al*, 1989). After paternal exposure to cyclophosphamide, mitomycin C or procabazine in mice, genomic instability is elevated in the bone marrow and germ line of progeny (Glen and Dubrova, 2012). Transgenerational effects of doxorubicin have been reported following exposure in female mice, with an increase in neonatal death, physical malformations and chromosomal abnormalities in the fourth and sixth generation (Kujjo *et al*, 2011)

7.2.3.5. Proposed experimental model

In human patients, it is the primordial follicle reserve which is the most important consideration for mutagenesis experiments as it is these follicles that will provide the germ cells for possible offspring. Exposing primordial follicles to chemotherapy agents and then later investigating their developmental competence would therefore be the most applicable model to determine if chemotherapy has a mutagenic effect on germ cells. Within the experimental design of previous chapters, primordial follicles in neonatal mouse ovaries were exposed *in vitro* to chemotherapeutic agents and in that culture, they develop to the primary/secondary stage. Developmental competence can only be explored if mature, fertilisable oocytes could be obtained from these primary/secondary follicles. One way of achieving this would be to use the culture system designed by Eppig and colleagues in which oocyte-granulosa cell complexes are obtained from cultured whole neonatal ovaries and then matured using a different culture paradigm. Maturing primary follicles to fertilisable oocytes in the mouse is an experimental technique which has been established in other laboratories (Eppig and O'Brien, 1996; O'Brien et al., 2003) but not in our own. Preliminary experiments using this technique have been unsuccessful in our laboratory and so a new experimental approach is being undertaken. Grafting of newborn ovaries into adult mice is being explored in our laboratory as a potential alternative approach for maturing primordial follicles following exposure to chemotherapeutic drugs. Grafting of ovarian material has proven to be a useful tool, with ovarian material from various species being xenografted into immunodeficient mice (Gosden et al., 1994; Senbon et al., 2005) as well as transplantation of mouse ovarian material back into mice, and its subsequent successful development (Li et al., 2010). Autografting

of cryopreserved ovarian tissue in women is used clinically and live births have been reported (Donnez et al., 2012). The location of grafting is a consideration, with the bursal cavity, the kidney capsule and subcutaneous sites all described (Bols et al., 2010). The location of the grafted material may directly influence how many oocytes are recovered as well as their quality (Yang et al., 2006). The kidney capsule is an attractive prospect as it provides a good blood supply (Gosden et al., 1994) and there is evidence that more oocytes are recovered from grafts under the kidney capsule when compared to subcutaneous locations (Hernandez-Fonseca et al., 2004).

For oocyte recovery, animals will be treated with hCG eight hours before collection of the ovaries (Li et al., 2010). Large follicles will then be punctured and the oocyte-cumulus complexes collected for IVF. Utilising embryo transfer, blastocysts could be used to produce offspring which could be monitored for epigenetic modifications and other abnormalities. Subsequent mating of these animals could provide information on any transgenerational changes caused by the chemotherapeutic agents.

A very recent study utilised a similar technique in order to explore if imatinib could protect oocytes from cisplatin induced damage and exposed neonatal ovaries before grafting them under the kidney capsule (Kim *et al*, 2013). However, this study did not go as far as the experiment proposed here and attempt to fertilise the oocytes, and so fully examine their competence. An experimental system such as the one described here could potentially be a useful tool to examine the effects of various chemotherapeutic agents on the competence of oocytes when exposed at the primordial stage, as well as determining the ability of protective treatments to successfully prevent any damage. In my opinion, this is the most important next step following the work undertaken in this PhD thesis.

7.2.4. Targeted chemotherapy treatments and personalized medicine

New drug treatments which are more effective anti-cancer agents than those described in this thesis and which are better tolerated by patients are in high demand and the subject of much research. A more personalized approach to cancer treatment is becoming a reality, with the sequencing of various cancer genomes meaning that new drugs and regimes can be tailored specifically to the patient (Yarden *et al*, 2013). As our knowledge of cancer biology increases, drugs that specifically exploit the differences and vulnerabilities of tumour cells can be developed. The identification of novel mutations and biochemical changes in tumour cells also opens up the possibility of directly localising drug action to the cancer itself. As discussed above, emerging technologies such as nanoparticles could also aid in the more specific localisation of a chemotherapy agent to a tumour site. The model which has been used here could remain a useful tool, in investigating the adverse effects of new drug treatments on female fertility as they become available. However, it could be that with more targeted treatment, adverse side effects such as POF will not be such a problem in the future.

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APPENDIX A. LIST OF SUPPLIERS

BD	Oxford, UK
Bio-Rad	Hemel Hempstead, UK
Camlab	Cambridge, UK
Dako UK Ltd	Ely, UK
Fisher Scientific	Loughborough, UK
GraphPad Software, Inc.	California USA
Greiner Bio-One	Stonehouse, UK
Invitrogen	Paisley, UK
Iwaki	through Sterilin Ltd, Aberbargoed UK
Leica UK	Milton Keynes, UK
LI-COR Biosciences, Ltd.	Cambridge, UK
Molecular Probes	see Invitrogen
New England Biolabs	Hitchin, UK
Nikon	Tokyo, Japan
Promega	Southampton, UK
Quiagen	Crawley, UK
Roche	Lewes, UK
Sakura Fintek UK Ltd	Thatchem UK
Sigma Aldrich Ltd	Poole, Uk
Thermoscientific	see Fisher Scientific
Vector	Peterborough, UK
VWR	Lutterworth, UK
Zeiss	Hertfordshire, UK

APPENDIX B. PUBLICATIONS

The effect of cisplatin on the ovary.

Stephanie Morgan, Richard Anderson and Norah Spears.

Centre for Integrative Physiology and *Centre for Reproductive Health, University of Edinburgh

Presented at Fertility, 2010.

Treatment of premenopausal women with chemotherapeutic agents can lead to germ cell loss and hence premature ovarian failure, but the exact mechanism by which this loss occurs is not well understood. Cisplatin is a DNA cross-linking agent commonly used in the treatment of lymphomas, sarcomas and germ cell cancers. Cisplatin was administered to several *in vitro* ovary culture models to identify the precise sequence of events which ultimately lead to germ cell loss. The earliest stages of follicle development (primordial, transitional and primary) were examined using newborn mouse ovaries cultured *in vitro*. After 24 hours in the presence of cisplatin, newborn ovaries exhibited a significant increase in protein expression of the apoptosis marker cleaved PARP, detected by Western blotting. After 6 days in the presence of varying concentrations of cisplatin, histological examination showed follicles with oocyte-specific damage: furthermore only primary stage follicles were affected (up to 45% compared with 7% in controls). Bovine ovaries were also cultured since they, like human ovaries, have dense stromal ovarian tissue. Bovine cortical strips cultured *in vitro* for 6 days with varying doses of cisplatin showed oocyte-specific cell death, similar to the murine data. We have found using a variety of other drugs that other chemotherapeutic agents (doxorubicin, docetaxel) initially damage granulosa cells,

with germ cell loss a downstream consequence; the results here indicate that in contrast, cisplatin selectively acts on the oocyte.

The effect of cisplatin and doxorubicin on the ovary

Stephanie Morgan, *Richard Anderson and Norah Spears

Centre for Integrative Physiology and *Centre for Reproductive Health, University of Edinburgh

Presented at Reproductive Function and Dysfunction, 2011.

Chemotherapy treatments cause germ cell loss, and hence premature ovarian failure, in premenopausal women, but the precise mechanism by which this occurs is unclear. Cisplatin is a DNA cross-linking agent commonly used in the treatment of lung, ovarian and bladder cancers, while the anthracycline doxorubicin is commonly used to treat breast cancer and leukaemia. Here, cisplatin and doxorubicin were administered to an *in vitro* ovary culture model to identify the precise sequence of events occurring in the ovary which ultimately leads to germ cell loss. The earliest stages of follicle development (primordial, transitional and primary) were examined using newborn mouse ovaries cultured *in vitro*. Ovaries were cultured for 6 days in total and exposed to either drug in varying doses during Day 2 of culture. In cisplatin-treated ovaries, histological examination showed follicles with oocyte-specific damage: furthermore only primary stage follicles were affected (up to 98% compared with 13% in controls, $p < 0.001$). In contrast, doxorubicin treatment caused damage specifically to granulosa cells and affected all stages of follicle present. Apoptosis in tissue was examined by determining expression of cleaved PARP by Western blotting, with tissue collected at the end of Day 2 of culture. Both drug treatments significantly increased expression of cleaved PARP but the effect of cisplatin was over 4-fold greater. These data indicate that cisplatin and doxorubicin

cause follicle loss through different mechanisms, suggesting that treatment of chemotherapy patients to try to prevent ovarian damage may need to be targeted to the drug(s) involved.

Cisplatin and Doxorubicin cause ovarian cell death through apoptosis not autophagy.

Stephanie Morgan, Richard A. Anderson and Norah Spears.

Centre for Integrative Physiology and *Centre for Reproductive Health, University of Edinburgh

Presented at the Society for the Study of Reproduction meeting, 2013

Chemotherapy treatment in premenopausal women has been linked to follicle loss and premature ovarian failure but the precise mechanisms of this are unclear.

Cisplatin is a DNA cross-linking agent commonly used in the treatment of ovarian, lung and bladder cancers, while the anthracycline doxorubicin is commonly used to treat leukaemia and breast cancer. The aim of this work was to compare the action of these two drugs in an *in vitro* ovary culture model and to establish the molecular mechanism underlying cell damage. Newborn mouse ovaries were cultured for 24 hours before being exposed *in vitro* to 5µg/ml cisplatin or 0.2µg/ml doxorubicin for a further 24 hours. Quantitative PCR analysis of these ovaries showed no expression of autophagy genes *Atg7*, *Atg13* or *Ulk1*. In addition, expression of *Beclin1* was reduced with both drug treatments (4-fold reduction for cisplatin when compared to control, $p=0.003$, 2 fold reduction for doxorubicin, $p=0.015$, $n=3$). To provide further evidence that apoptosis rather than autophagy was the predominant cell death pathway, immunohistochemical analysis was carried out for two apoptosis markers: cleaved PARP and cleaved caspase 3. The drug concentration was reduced for this analysis to 0.5µg/ml cisplatin and 0.05µg/ml doxorubicin, as at the higher doses, the damage was too widespread. There was a significant increase in the number of

cells/mm² staining positive for cleaved PARP following doxorubicin treatment (Mean=332±57 for doxorubicin compared to 173±20 for control, p=0.005, n=6). Analysis of the affected cell types showed that doxorubicin caused a significant increase in the number of stromal (Mean= 273±60 compared to 118±19 in control, p=0.009) and granulosa cells (Mean= 30±6 compared to 11±3 in control, p=0.011) staining positive, but there was no change in the number of oocytes expressing cleaved PARP indicating that doxorubicin causes follicle loss primarily through apoptosis of ovarian somatic cells. The expression of cleaved caspase 3 also revealed a significantly higher number of granulosa cells/mm² staining positive following treatment with doxorubicin (Mean= 65±20, compared to 8±4 in control, p=0.05, n=5) but as with cleaved PARP there was no change in oocyte expression. Cisplatin caused a non-significant increase in somatic cells/mm² staining positive for cleaved PARP (Mean= 238±23 compared to 173±20 in control, p=0.063, n=6) and there was no significant increase in the number of oocytes staining positive for either cleaved PARP (Mean= 104±23 compared to 73±8 in control, p=0.271) or cleaved caspase 3 (Mean=55±11 compared to 32±7 in control, p=0.246) following cisplatin treatment, although there was a trend towards an increase for both markers. This work shows that doxorubicin causes ovarian cell damage through apoptosis rather than autophagy, and that the primary site of cell damage is the somatic cells, rather than the oocyte. The locus and mechanism of follicle loss with cisplatin is unclear from these data, but it appears to be through different pathways to doxorubicin.